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The Prediction of Oxygen Transfer Rates in the Presence of Mould Mycelium

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Summary. An empirical relationship of oxygen transfer rate and mould viscosity is discussed, which leads to equations for predicting the OTR of mould fermentations.

Introduction

Chain *et al.*[2] found that the oxygen transfer rate (OTR) of a fermentor is reduced by mould mycelium. This effect was examined quantitatively by Deindoerfer and Gaden,[4] who showed that the OTR is a function of broth rigidity.

Solomons[6] showed that the OTR of a fermentor of fixed design can be predicted although this applies only to the performance in sulphite solution (Cooper, Fernstrom and Miller[3]). We have now re-examined the polarographic data of Solomons and Perkin,[8] and as modified this enables a prediction of the OTR of a 1-litre fermentor to be made under any given conditions of mycelium concentration and stirrer speed.

Experimental

The vessel was fully baffled and of 1-litre capacity with a diameter : height ratio of 1 : 1. The impeller, centrally mounted, was a six-bladed disc turbine 50 per cent of the diameter of the vessel. Air was sparged at the rate of 1 vol. per vol. of media per min through a single-hole sparger mounted on the bottom of the vessel. *Aspergillus* mycelium was grown, washed several times with distilled water and suspended in the final solution (0·1 M KCl) with 10^{-3} M sodium azide to inhibit any respiration. The Bingham plastic mould viscosity was examined with a Ferranti

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VM viscometer and a plot of apparent viscosity *versus* shear rate was extrapolated to 1 sec^{-1} (not 1 dyne cm^{-1} sec^{-1} as mis-stated in the original paper). Suspensions of 100, 1,000 and 10,000 cP were prepared and stirred at 400, 600, 800, 1,000 and 1,200 rev/min. The polarographic gassing-out method of Wise[10] was used with a vibrating platinum cathode and $Ag/AgCl_2$ anode as described by Chain *et al.*[2] The temperature was controlled at $20° \pm 0{\cdot}5°$.

Results

The original graph (Solomons and Perkin[8]) of OTR *vs.* stirrer speed is shown in Fig. 1. If OTR is plotted against the log of the

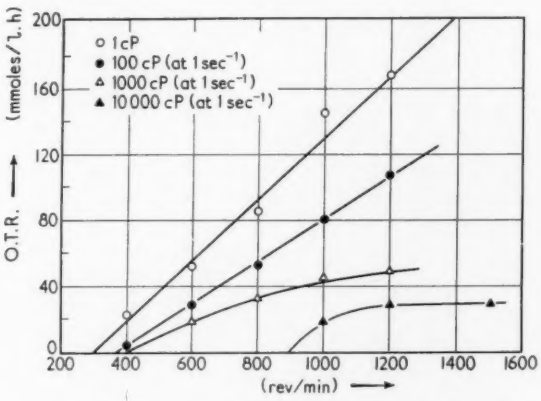

Fig.1. Effect of stirrer speed on oxygen transfer rates

viscosity for each stirrer speed, a series of straight lines is obtained, all of which meet at a single point (Fig. 2). Furthermore, the slope of the line for a given stirrer speed is a linear function of that speed (Fig. 3). A logarithmic plot of viscosity against mould concentration is shown in Fig. 4.

Hence we have the relationships:

from Fig. 2: $\text{OTR} = K_n(\log \mu - 5{\cdot}175) - 10{\cdot}94$ mmoles/l. h
from Fig. 3: $K_n = 8{\cdot}6 - 0{\cdot}036\,N$
from Fig. 4: $\log \mu = 2{\cdot}65 \log C$

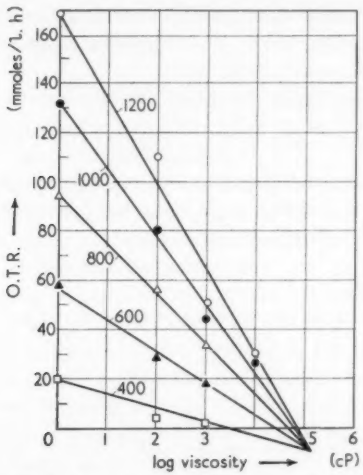

Fig. 2. Effect of mould viscosity on oxygen transfer rates

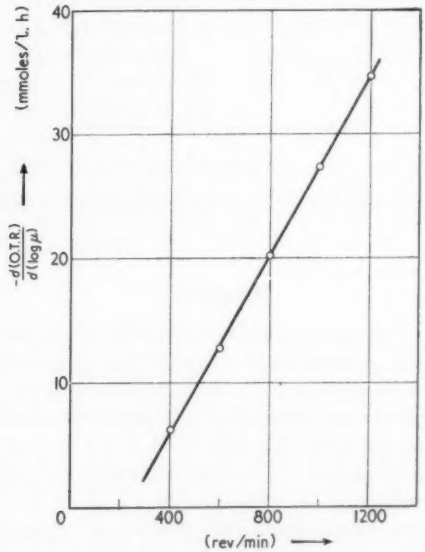

Fig. 3. Rate of change of OTR with viscosity related to stirrer speed

where K_n = constant for given N and is $-d(\text{OTR})/d(\log \mu)$; μ = mould viscosity, cP; N = impeller speed, rev/min; and C = mycelium concentration, g/l.

From these three linear relationships, we obtain the expression:

$$\text{OTR} = (0{\cdot}036N - 8{\cdot}6)\,(5{\cdot}175 - 2{\cdot}65 \log C) - 10{\cdot}94 \text{ mmoles/l. h}$$

Knowing the relationship between mycelium concentration and broth viscosity, the OTR may thus be predicted for any given stirrer speed and mould concentration.

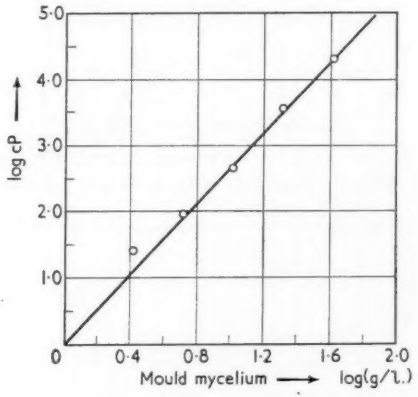

Fig. 4. Relationship between mould concentration and broth viscosity

Discussion

In the case of Bingham plastic viscosities of the magnitudes discussed here, the extrapolated value of the apparent viscosity at 1 sec^{-1} may be considered to be the yield stress of the fluid. However, since the characteristics of a Newtonian fluid of viscosity 1 cP give compatible results, the OTR must be controlled by the rigidity of the medium. This suggests that the yield stress of an *Aspergillus* suspension is numerically equal to its rigidity; this relationship was demonstrated in the case of *Penicillium* suspensions by Deindoerfer and Gaden.[4]

It may be found that the OTR is a function of impeller shear according to the relationship:

$$\text{shear rate sec}^{-1} = 10N$$

found by Calderbank and Moo-Young.[1] This would need examining on a range of vessel sizes.

There must, however, be a relationship between impeller power (expressed as shear) and viscosity breakdown. Otto[5] presents a graph from the work of Taylor[9] which shows how rapidly shear rate falls off with distance from the impeller tip. In view of this, we consider that in viscous mould broths virtually all of the oxygen transfer takes place within the impeller envelope; little appears to be contributed by the bubbles in the mass of the liquid outside the envelope since they are, in the main, very slow-moving. Thus, in a given fermentor, one is only able to aerate broths adequately below a certain viscosity, and this appears to be connected with vessel size and impeller pumping capacity as discussed by Solomons.[7]

Chain *et al.*[2] and Solomons and Perkin[8] found that for concentrations of mould mycelium above a certain value, increase in stirrer speed produces little increase in turbulence and the additional power is simply dissipated as heat. This effect is apparent on examination of Fig. 2.

Acknowledgements. We wish to acknowledge the permission of the Editor of the *Journal of Applied Chemistry* to reproduce Fig. 1. We also thank the Directors of The British Drug Houses Limited for permission to publish this work and in particular Dr. C. Ockrent for his advice on its presentation.

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Cation Exchangers on a Sugar-beet Pulp Base. Application for Decontaminating Radioactive Waste Water

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Summary. A cation exchanger suitable for decontaminating low and medium radioactive waste water was sought. Regeneration being considered undesirable, the exchange material had to be cheap and readily obtainable. Sugar-beet pulp, a weakly acidic cation exchanger, satisfies these conditions. Its capacity is about 0·62 mg eq. per gram of dry matter. In order to study the selectivity of the sugar-beet pulp exchanger, the equilibrium curve of the reaction

$$2\,RNa + Sr^{++} \rightleftharpoons R_2Sr + 2\,Na^+ \qquad (R = \text{ion exchanger})$$

was determined.

This curve was found to have the shape of the hyperbola proposed by Waterman and Weber for the characterization of the course of simultaneous reactions. Sugar-beet pulp adsorbs the salts of the alkaline earth metals selectively in the presence of both Na^+ and La^{+++}. Sugar-beet pulp was used to decontaminate a solution containing $^{140}BaCl_2$ and $^{140}LaCl_3$ and having an activity of about 10^{-2} μc/ml. The results were satisfactory.

By treating sugar-beet pulp with formaldehyde and hydrochloric acid, the amount of water bound to the pulp is very much decreased. By this treatment a cation exchanger is produced having a capacity per unit volume about six times greater than that of sugar-beet pulp. The capacity of this exchanger is about 0·5 mg eq. per gram of dry matter. The selective behaviour of sugar-beet pulp treated with formaldehyde is similar to that of unmodified sugar-beet pulp.

By treating sugar-beet pulp with formaldehyde, hydrochloric acid and dilute sulphuric acid, a cation exchanger with very good mechanical and filtration properties is obtained. This exchanger has a capacity of about 1·3 mg eq. per gram of dry matter.

It seems possible to employ the cation exchangers obtained by treatment of sugar-beet pulp with either formaldehyde and HCl or formaldehyde, HCl and H_2SO_4 also for purposes other than the removal of radioactive cations from water.

Introduction

In some ion exchange processes, it is undesirable for the ion exchanger to be regenerated after use. The decontamination of radioactive waste water by ion exchange is such a process. Ion exchangers are used for decontaminating low and medium radioactive waste water, that is water with an activity of less than about 0·1/μc/ml.[18, 19] This kind of waste water is produced in large quantities in nuclear fuel processing plants. Sources of this radioactive waste water are, for instance, the later stages of processes for separating fission products from nuclear fuels; primary cooling water from certain types of nuclear reactors; water used for decontaminating buildings, plant equipment or clothes; and laboratory samples and wastes.[14, 19] The waste water must, of course, be almost completely free from radioactive substances before it leaves the plant. Radioactive contaminants have to be removed from the water in such a way that a radioactive concentrate is obtained which can be stored safely and without occupying too much room. Ion exchangers can be advantageously used in decontaminating radioactive waste water because the concentrate of active ions obtained is solid and therefore relatively easy to handle. By regeneration of the exchange material this advantage is lost again.

Ion exchangers used in processes where they are not regenerated must be very cheap. This renders organic synthetic exchange resins unattractive for decontaminating radioactive waste water. Therefore much attention has been paid to the development of inexpensive ion exchangers. A number of naturally-occurring exchange materials, both inorganic (*viz.* various clays[14]) and organic, have been investigated. The advantage of using an organic ion exchanger is that the material can be burned after having been charged with radioactive matter. As a result, the radioactive wastes are obtained in a highly concentrated form and the costs of final storage are appreciably reduced. On the other hand, it is expensive to burn radioactive materials safely. According to McEwen *et al.*,[13] the incineration costs are 63·5 U.S. dollars/m^3 of dry substance. However, more recent literature data[12] seem to indicate that these costs can be reduced. The most important investigations into the properties of

naturally-occurring ion exchangers are those conducted by D'Hont, Dejonghe and co-workers.[3–5] These authors used sawdust, rotted sawdust and lignite. The exchange properties of these materials are satisfactory, but they have some drawbacks. The filtration properties of sawdust are irregular, while lignite is peptised by alkaline solutions. Smallwood[20] investigated the ion exchange properties of activated sludges obtained in waste-water purification installations. As was to be expected, the capacities of the activated sludges proved to be very low.

In the present paper, the cation exchange properties of sugar-beet pulp are described. The advantage of sugar-beet pulp is that it is a waste material. In the Netherlands it is obtained in large quantities at relatively low prices, either fresh or dried. Its main components are cellulose, denatured proteins and pectin. Judging from the experiments of Dejonghe one might expect cellulose to be a cation exchanger of low capacity. Proteins can also act as ion exchangers at certain pH values. It seems obvious, however, that the cation exchange properties of sugar-beet pulp are mainly due to the presence of pectin.

Pectin is a naturally-occurring polymer of α-D-galacturonic acid in which a number of carboxyl groups have been esterified with methanol. As the dissociations of the different carboxyl groups in the polymer influence each other, the dissociation constant K_z of pectin is found to possess no definite value, but a range of values. Speiser *et al.*[21] found a range of K_z values from $3{\cdot}30 \times 10^{-4}$ to $1{\cdot}23 \times 10^{-5}$ for a pectin in which 10 per cent of the carboxyl groups present were esterified. The well-known pectin investigator Deuel and his school have proved beyond doubt[6–10] that both pure pectin and pectin treated with formaldehyde are cation exchangers. In the presence of alkali metal ions, pectin selectively adsorbs the ions of alkaline earth metals.

Some investigators have found that sugar-beet and sugar-beet pulp possess cation exchange properties. Van der Poel[17] observed a strong increase in the loss of mineral constituents from sugar-beet during the extraction of sugar when the pH of the water used for extraction fell below 5. This observation is in accordance with those made by Speiser *et al.*,[21] described above. Briggs *et al.*[1] observed that discs cut out of sugar-beet possess ion exchange properties. Carruthers and Oldfield[2] found that

sugar-beet pulp is able to exchange Ca^{++} for Na^{+} and NH_4^{+}. By feeding sugar-beet pulp and other ion exchangers to rats injected subcutaneously with $^{134}CsCl$, ^{42}KCl or $^{86}RbCl$, Mraz and Patrick[15] and Williams and Patrick[25] found that there exists affinity between the cations of these salts and sugar-beet pulp. Mraz and Patrick[15] also observed this affinity during experiments *in vitro*. None of the authors mentioned seems to have hit on the use of sugar-beet pulp as an ion exchanger.

In the present investigations Sr^{++} was mostly used as the cation to be adsorbed. It was chosen because salts of radioactive strontium, for instance ^{90}Sr, frequently appear in radioactive waste waters. This isotope is well known to be very dangerous to health, owing to its long radioactive and biological half-life periods. For reasons of safety and lack of accurate γ-ray measuring apparatus, no isotope of Sr could be used in the experiments with a radioactive cation. Therefore Sr was replaced by ^{140}Ba in these experiments.

By modifying sugar-beet pulp, cation exchangers were prepared having properties superior to those of sugar-beet pulp itself. Possibly these exchange materials can also be employed for purposes other than the decontamination of radioactive waste water.

Capacity of Sugar-Beet Pulp

Two kinds of sugar-beet pulp were used:

(1) Dried sugar-beet pulp, obtained by the Steffen process. In the Steffen process the beet cells are killed thermally. Dried sugar-beet pulp was used in most experiments as this material can be stored for long periods without deterioration. This makes it possible to exclude the influence of variations in pulp quality during a series of experiments.

(2) Fresh sugar-beet pulp, obtained in the 'cold diffusion' process developed in the laboratory at Delft.[17] In this process, the sugar-beet cells are killed by means of chloroform in the liquid phase, water of 20°–40°C being employed for the sugar extraction.

The ion exchange experiments were carried out in columns with a diameter of 40 mm. The size of fresh sugar-beet pulp particles obtained in the 'cold diffusion' process was reduced to about 1 mm in order to prevent wall effects. In comparative

experiments, the shapes of the break-through curves showed that surprisingly small wall effects occur when dried pulp obtained in the Steffen process is used. This we probably owe to the rather soft and plastic Steffen pulp's being easily pressed firmly against the column wall. The filtration properties of Steffen pulp are satisfactory: a flow rate of 2·0 cm^3/cm^2 min can readily be maintained. At appreciably higher flow rates the pulp is squeezed together and clogs the column. Fresh pulp obtained in the 'cold diffusion' process is a much firmer material than Steffen pulp. Its filtration properties being better, higher flow rates can be used.

In sugar-beet pulp obtained by the Steffen process, about half the ion exchange sites proved to be occupied by Mg^{++} and Ca^{++}. A pretreatment with dilute acid is therefore necessary if it is desired to use the full capacity of the pulp. In both kinds of sugar-beet pulp studied, all esterified carboxyl groups were found to have been saponified already during the sugar extraction.

The capacity of sugar-beet pulp was determined by the column method, proposed by Klamer.[11] A column was filled with pulp in the H-form. A solution of a cation of known concentration was then passed through the column till the composition of the effluent had become equal to that of the inflowing liquid. As sugar-beet pulp is a weakly acid ion exchanger (carboxyl groups), the capacity is dependent on the pH; therefore a buffer solution had to be used. In the experiments with Sr^{++} the buffer chosen was $Sr(HS)_2$4aq. This salt was prepared according to Terres and Brückner[22] by passing a stream of H_2S gas into a suspension of $Sr(OH)_2$ in water at 65°–70°C.

After the column had been saturated with Sr^{++} the $Sr(HS)_2$ solution was washed out of the pore volume with distilled water. The column was then regenerated with a dilute solution of HCl. The regenerated Sr^{++} was determined complexometrically according to Van Nieuwenburg and van Ligten,[16] using fluorexon as indicator. After the regeneration, the amount of dry matter in the column was determined by removing the pulp from the column and drying to constant weight at about 100°C. In corresponding determinations of the capacity of sugar-beet pulp for Na^+ ions the buffering salt chosen was $NaHCO_3$.

The results of the determinations of the capacity are given in Table I. Notwithstanding the relative inaccuracy of the Na^+

determination, the Na^+ capacity of the pulp is mentioned in this table to show that the order of magnitude of the ion exchange capacity for Sr^{++} and Na^+ is the same.

Table I. Capacities of sugar-beet pulp for Sr^{++} and Na^+ ions (sugar-beet pulp obtained by the Steffen process: column diameter 40 mm; flow rate about 1 cm^3/cm^2 min)

Salt used	Concentration of cation in inflowing solution, mg eq./ml	pH of effluent at equilibrium	Capacity of pulp, mg eq./g of dry matter
$Sr(HS)_2$	$11{\cdot}3 \times 10^{-3}$	8·6	0·63
$Sr(HS)_2$	$5{\cdot}7 \times 10^{-3}$	8·4	0·66
$Sr(HS)_2$	$2{\cdot}8 \times 10^{-3}$	8·2	0·62
$NaHCO_3$	$1{\cdot}0 \times 10^{-2}$	7·2	0·52

The capacity for Sr^{++} ions of sugar-beet pulp obtained by the 'cold diffusion' process was determined in the same way. As the value found was 0·62 mg eq./g of dry substance, no difference exists between the capacities of the two kinds of pulp.

Sugar-beet pulp remains insoluble even at pH values higher than 8. Initially the effluent from a column filled with Steffen process pulp has a pale green colour at pH values higher than 7. This colour is probably due to a green dye being extracted from the pulp. The soluble component of the pulp contains no Sr^{++}. No dyes are extracted from sugar-beet pulp obtained by the 'cold diffusion' process.

Selectivity

As is to be expected of a weakly acidic ion exchanger, the capacity of sugar-beet pulp depends strongly on the pH. At pH 3 the capacity of the pulp is very low. This observation is in accordance with the range of K_z values between 10^{-4} and 10^{-5} found by Speiser *et al.*[21] During the exchange reaction:

$$2RH + Sr^+ \rightleftharpoons R_2Sr + 2H^+ \qquad (R = \text{ion exchanger})$$

the pH decreases, causing a drop in capacity of the cation exchanger. If it is desired to remove Sr^{++} from a concentrated solution of this ion, a buffering salt like $Sr(HS)_2$, mentioned above,

must be chosen. In waste water, Sr^{++} will, of course, not generally be present in the form of a buffering salt. From investigations by Deuel, Hutschneker and Solms,[8] pectin is known to adsorb Sr^{++} ions selectively in the presence of Na^{+} ions. If sugar-beet pulp has the same property it should be possible to avoid difficulties arising from a decrease in the pH by employing the pulp not in the H-form, but in the Na-form.

For this reason the present authors studied the exchange equilibrium

$$2RNa + Sr^{++} \rightleftharpoons R_2Sr + 2Na^{+} \qquad (1)$$

The equilibrium curve of this reaction was determined by the use of a method proposed by Wevers.[24] In this case a column filled with sugar-beet pulp obtained by the Steffen process was saturated with Sr^{++} at pH 5.

The column was then percolated with a $2{\cdot}5 \times 10^{-3}$ N solution of sodium acetate that had been acidified with HCl till pH 5. The points of the equilibrium curve that were determined have been plotted in Fig. 1. The points were correlated by the method of Waterman and Weber.[23]

According to Waterman and Weber, the course of simultaneous reactions can often be described by a hyperbola of the shape

$$y = x + \frac{x(1-x)}{a+bx} \qquad (2)$$

where in the present case

$$y = \left[\frac{\text{g eq. } Sr^{++}/\text{litre}}{(\text{g eq. } Sr^{++} + \text{g eq. } Na^{+})/\text{litre}}\right] \text{ bound to exchanger}$$

$$x = \left[\frac{\text{g eq. } Sr^{++}/\text{litre}}{(\text{g eq. } Sr^{++} + \text{g eq. } Na^{+})/\text{litre}}\right] \text{ in solution}$$

and a and b are characteristic parameters.

The hyperbola according to Waterman and Weber, represented by equation (2), has also been plotted in Fig. 1. The parameters a and b were calculated by the method of least squares from the points of the equilibrium curve determined experimentally. It was found that:

$$a = 0{\cdot}0555; \qquad b = 1{\cdot}035$$

It appears that the equilibrium curve can be represented very accurately by a hyperbola. The ability of the sugar-beet pulp exchanger to remove Sr^{++} from a solution containing Na^{+} can now either be read from Fig. 1 or calculated from equation (2).

Sugar-beet pulp is seen to adsorb Sr^{++} very selectively in the presence of Na^{+}. This observation is in accordance with the results obtained by Deuel and co-workers. If, in the removal of Sr^{++} from a solution by means of sugar-beet pulp, a drop in pH causes difficulties, the pulp can be applied in the sodium form.

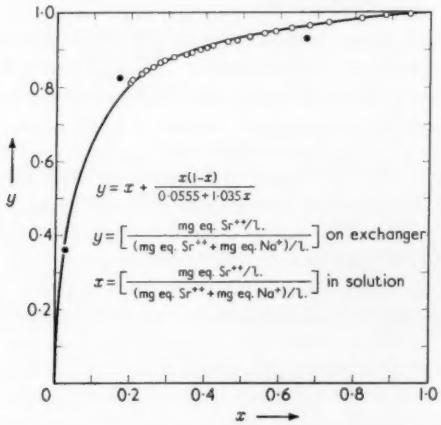

Fig. 1. Selectivity diagram according to Waterman and Weber[23] for

$$2RNa + Sr^{++} \rightleftharpoons R_2Sr + 2Na^{+}$$

○ = experiments with sugar-beet pulp
● = experiments with sugar-beet pulp modified with formaldehyde
($Sr^{++} = 10^{-2}$ mg eq./ml)

Experiments with Radioactive Isotopes

In low and medium radioactive waste water the radioactive contaminants are present in very low concentrations. A solution having an activity of 10^{-2} μc/ml and containing no radioactive contaminants but ions of ^{90}Sr has a Sr^{++} concentration of $1{\cdot}3 \times 10^{-9}$ N. The authors therefore studied the ability of sugar-beet pulp to remove cations from water in which the ions are present in

extremely low concentrations. Chemical analysis of cations in these concentrations obviously being impossible, use had to be made of radioactive isotopes. As mentioned in the introduction, Sr had to be replaced by ^{140}Ba in these experiments. The radioactive decay of ^{140}Ba follows the equation:

$$^{140}Ba \xrightarrow{\beta,\gamma} {}^{140}La \xrightarrow{\beta,\gamma} {}^{140}Ce.$$

^{140}Ba has a half-life period of 12·8 days, ^{140}La one of 40·4 h; ^{140}Ce is not radioactive. A glass column with a diameter of 40 mm was filled to a height of 38 cm with sugar-beet pulp obtained by the Steffen process. The column was percolated with 6 l. of a solution of a radioactive equilibrium mixture of $^{140}BaCl_2$ and $^{140}LaCl_3$ in distilled water. The total activity of the solution was about $0{\cdot}8 \times 10^{-2}$ μc/ml. Samples of 0·3 ml were taken from this solution. The effluent was collected in 1-l. volumetric flasks. From each flask two samples of 0·6 ml were taken. The samples were evaporated and the activity was determined in a Geiger–Müller counter equipped to measure β-radiation. The activities measured were corrected for the time elapsed since the taking of the sample. In the case of the samples from the effluent, this last correction was made on the assumption that the radioactive material that had passed through the exchanger bed consisted wholly of La^{+++}. The decontamination was calculated from the loss of radioactivity through the ion exchange process and defined as

$$\text{Decontamination} = \frac{A-B}{A}\,100\%$$

where:

A = activity of inflowing solution expressed in disintegrations per unit volume per unit time,

B = activity of effluent, expressed in disintegrations per unit volume per unit time.

Results are given in Table II.

From the rate of radioactive decay in the effluents it was found that the radioactive material that passes through the ion exchanger consists mainly of La^{+++}.

Sugar-beet pulp appears to adsorb Ba^{++} selectively in the presence of the trivalent ion La^{+++}.

Table II. Decontamination of a solution of $^{140}BaCl_2$ and $^{140}LaCl_3$; column diameter 40 mm, column height 38 cm; total activity of inflowing solution about $0{\cdot}8 \times 10^{-2}$ μc/ml; ^{140}Ba and ^{140}La in radioactive equilibrium

Sample taken from *n*th litre of effluent from column, *n*	Decontamination calculated from loss of radiation, %	pH of effluent	Flow rate, cm^3/cm^2 min
3	98·8	4·2	1·6
4	98·9	4·2	1·9
5	99·3	4·2	2·0
6	99·3	4·2	1·9

[a] Decontaminations in first two litres of effluent are not reliable as these may contain distilled water from original pore volume of exchanger.

After 6 l. of solution had been passed through the exchanger, the column was scanned from the outside with a monitor equipped to measure γ-radiation. It was found that considerable amounts of radioactive contaminants had penetrated into the exchanger bed over a length not exceeding 5 cm.

Sugar-beet Pulp Modified with Formaldehyde

A disadvantage of the use of sugar-beet pulp is the large amount of water bound by this material. The sugar-beet pulp exchanger was found to contain only 0·04–0·05 g of dry matter per cm^3 of column volume. Rather large column volumes are required for the industrial application of a sugar-beet ion exchanger.

It is a well-known fact that cellulose and proteins can be made less hydrophilic by treatment with formaldehyde. Deuel[6] has found that during the reaction between formaldehyde and pectin methylol groups are formed:

$$ROH + H_2CO \rightarrow ROCH_2OH$$

Such a methylol group reacts with an —OH group from another pectin molecule according to

$$ROCH_2OH + HOR' \rightarrow ROCH_2OR' + H_2O$$

Acids catalyse this reaction. If cross-linking occurs to a sufficient degree, an insoluble copolymer of pectin and formaldehyde is formed. As all main constituents of sugar-beet pulp are made less hydrophilic by the action of formaldehyde, the authors tried

to counteract the swelling of pulp with water by a formaldehyde treatment. Dried pulp (25 g) obtained by the Steffen process was soaked in a mixture of 100 ml of a 40 per cent formaldehyde solution and 100 ml 18 per cent HCl. After soaking, the excess liquid was removed as completely as possible by suction on a Büchner filter. The impregnated pulp was then dried at 110°C. Finally the modified pulp was washed with water. This is necessary because the first eluate from the modified pulp has a brown colour. When pulp treated in this manner is placed in water scarcely any swelling occurs. A column filled with a rather coarsely ground exchanger of this type contains 0·36 g of dry matter per cm^3, that is about eight times the solid substance content per unit volume of untreated pulp. The exchanger has good mechanical and filtration properties. The admissible flow rate is much higher than the rate that can be used in columns filled with unmodified pulp.

During the formaldehyde treatment, the capacity of the pulp decreases to 0·51 mg eq. per gram of dry matter at pH 6. Therefore the capacity per unit of exchanger volume is increased by a factor of about 6·5. At pH 4 the capacity of pulp treated with formaldehyde is still about 60 per cent of the capacity at pH 8.

Some experiments concerning the equilibrium

$$2RNa + Sr^{++} \rightleftharpoons R_2Sr + 2Na^+$$

were carried out with pulp treated with formaldehyde. In these experiments, the column was percolated with 0·01 N Sr^{++} solutions containing various amounts of sodium acetate until equilibrium had been reached.

The amounts of Sr^{++} adsorbed by the column at equilibrium were determined by regenerating the column and measuring the Sr^{++} content of the regenerate. The points of the equilibrium curve found in this way were once again plotted in Fig. 1. Curiously enough, the points are seen to lie rather close to the equilibrium curve given by the hyperbola according to Waterman and Weber, in spite of the fact that the hyperbola has been determined using unmodified pulp, while equilibrium has been reached in a different way and at a different concentration level. The formaldehyde treatment does not seem to have influenced the equilibrium very much.

Sugar-beet Pulp Modified with Sulphuric Acid and Formaldehyde

Ion exchangers are known to be produced by the treatment of some organic materials, e.g. sawdust, with sulphuric acid. The authors tried to combine the treatment of sugar-beet pulp with formaldehyde and HCl with a sulphuric acid treatment. Dried pulp (25 g) obtained by the Steffen process was soaked in a mixture of 50 ml 40 per cent formaldehyde, 50 ml 18 per cent HCl and 5 ml of concentrated sulphuric acid. After soaking, the excess liquid was removed as completely as possible by suction on a Büchner filter. The impregnated pulp was then dried at 110°C. During drying, some coking seems to occur. A product that does not swell with water and has excellent mechanical and filtration properties is obtained. The capacity is 1·30 mg eq. per gram of dry matter. Even the first eluate is completely colourless. It is interesting to note that in the first instance not concentrated but dilute sulphuric acid is applied in the production of this cation exchanger.

Other Experiments

Sugar-beet pulp was treated with dilute nitric acid. A product having undesirable filtration properties was obtained. Sugar-beet pulp was subjected to thermal treatment at 250°–300°C. The product obtained was dark and had a low capacity (0·13 mg eq./g of dry matter at pH 6).

Table III. Capacities of sugar-beet pulp, modified sugar-beet pulp and other cheap cation exchangers

Ion exchangers	Capacity, mg eq./g of dry matter	Ref.
Sugar-beet pulp	0·62	
Sugar-beet pulp, modified with formaldehyde and HCl	0·51	
Sugar-beet pulp, modified with formaldehyde and H_2SO_4	1·30	
Thermally treated sugar-beet pulp	0·13	
Lignite	0·4–0·6	3
Sawdust	0·15	4
Rotted sawdust	0·35	4
Activated sludge	0·001	20
Vermiculite	0·5	14
Montmorillonite	1·0	14

In Table III the capacities of cation exchangers mentioned in this paper are compared with those of exchangers that are more or less similar in character.

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Rapid Determination of 17-α-Oxy-11-desoxy-corticosterone in the Course of Biological Fermentative Oxidations

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Summary. A rapid analytical method has been developed to follow the biological oxidation of 17-α-oxy-11-desoxy-corticosterone (Reichstein compound S) to hydrocortisone. Paper chromatography is too slow to follow the progress of a preparative microbiological steroid transformation. However, 17-α-oxy-11-desoxy-corticosterone can be separated in a sufficiently pure state for photometric assay by a simple extraction from fermentation broths containing several steroids. This extraction, coupled with the formation of a sulphuric acid chromogen, permits estimation of the steroid in fermentation liquors. The steady decrease of the 17-α-oxy-11-desoxy-compound thus estimated proved to be a true indication of the progress of the oxidation process, allowing it to be stopped at the most favourable time.

Introduction

One possible way[1] of producing hydrocortisone is the microbiological oxidation of 17-α-oxy-11-desoxy-corticosterone (Reichstein compound S). When carried out in submerged cultures with various micro-organisms, this oxidation yields, besides hydrocortisone, the corresponding C_{11}-epimer (epi-hydrocortisone or epi-F); consequently the fermentation liquors contain three steroid compounds at the same time.

In order to be informed about the prevailing state of the transformation during a fermentative oxidation run, an adequate analytical method is needed. This method has to be rapid, and besides differentiating between the steroids present, it has to be insensitive to possible disturbing effects from organic components of the substrate.

Known methods[2,3] are generally based upon separation of the steroids by paper chromatography. These methods provide

trustworthy information about the transformations of the steroids, but since they are slow they delineate the stages of a process, going to completion within a few hours, after it is over. In the course of an earlier work,[4] also in connection with the biological oxidation of a steroid, it was possible to find a rapid method which obviates the lengthy chromatographic step, and is based upon selective extraction and a specific colour reaction. The same principle serves in the method presented here.

Experimental

Since the ultraviolet absorption curves of the three steroids are nearly identical (Fig. 1), attempts have been made to differentiate them by a suitable colour reaction. For this purpose the reaction of the steroids with concentrated sulphuric acid at room temperature promised most success.[5,6] To form the sulphuric acid chromogen, a solution in chloroform of the steroid is shaken with concentrated sulphuric acid. In this investigation, the three steroids were taken in identical concentrations (100 μg/ml) and in identical volumes (5 ml) of the chloroform solutions and shaken for 5 min with identical quantities (10 ml) of concentrated sulphuric acid; the absorption curves of the resulting chromogens are shown in Fig. 2.

The curves thus plotted, though showing some difference, were not suitable for evaluation because of their considerable superimposition. It thus became necessary to attempt the isolation from its mixture of one of the steroid components.

The distribution coefficients, K, of 17-α-oxy-11-desoxy-corticosterone and hydrocortisone were first determined for several solvent systems, together with the corresponding separation factors, β, the ratio of the distribution coefficients.[7] Some of the more characteristic values are presented in Table I. These show that the values of β, which is the measure of the separability of the two steroids, changed appreciably with changes in the composition of the solvent phases, and that quite high values ($\beta = 19{\cdot}5$) could be achieved. The experiments have indicated that the diminution of the alcohol content of the aqueous phase leads to high values of β, as does increase of the carbon tetrachloride content in the organic (non-polar) phase.

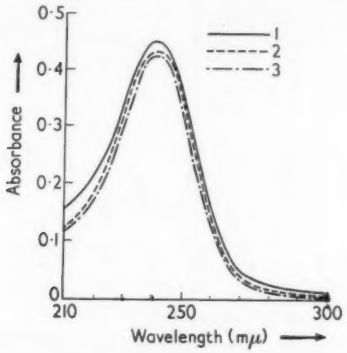

Fig. 1. Ultraviolet absorption spectra of 17-α-oxy-11-desoxy-corticosterone (1), hydrocortisone (2) and 11-epi-hydrocortisone (3) in absolute ethanol, 10-mm cuvettes. Concentration: 10 μg/ml

Following this, values of equilibrium concentrations have been measured when water was one of the phases, and the other phase was one of various mixtures of carbon tetrachloride and chloroform. Fig. 3 shows the content of 17-α-oxy-11-desoxy-corticosterone and hydrocortisone, respectively, in the organic phase when identical quantities of the steroids in equal volumes were equilibrated. From the curves, the optimum ratio (3 vols. : 1 vol.) of carbon tetrachloride to chloroform could be determined; with this mixture, the 17-α-oxy-11-desoxy-corticosterone could be extracted

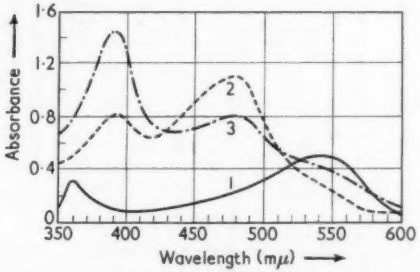

Fig. 2. Absorption spectra of the chromogens, formed with concentrated sulphuric acid, of 17-α-oxy-11-desoxy-corticosterone (1), hydrocortisone (2) and 11-epi-hydrocortisone (3) in 10-mm cuvettes. Concentration: 50 μg/ml

Table I. Partition ratios (K) and separation factors (β) of 17-α-oxy-11-desoxy-corticosterone and hydrocortisone in the case of different solvents

A phase	$EtOH–H_2O$ 75 v : 25 v	$MeOH–H_2O$ 75 v : 25 v	$EtOH–H_2O$ 50 v : 50 v	$MeOH–H_2O$ 50 v : 50 v	$MeOH–H_2O$ 40 v : 60 v	$MeOH–H_2O$ 50 v : 50 v	$MeOH–H_2O$ 50 v : 50 v
B phase	ClC_2H_4Cl	ClC_2H_4Cl	ClC_2H_4Cl	ClC_2H_4Cl	ClC_2H_4Cl	$ClC_2H_4Cl–CCl_4$ 75 v : 25 v	$ClC_2H_4Cl–CCl_4$ 50 v : 50 v
K_1	1·7	2·6	6·4	5·4	19·5	12·9	6·4
K_2	1·0	0·67	1·4	0·84	1·5	0·83	0·33
β	1·7	3·9	4·6	6·4	13·0	15·5	19·5

$$K_1 = \frac{C_1 \text{ in the B phase}}{C_1 \text{ in the A phase}} \qquad K_2 = \frac{C_2 \text{ in the B phase}}{C_2 \text{ in the A phase}} \qquad \beta = \frac{K_1}{K_2}$$

C_1 = 17-α-oxy-11-desoxy-corticosterone concentration

C_2 = hydrocortisone concentration

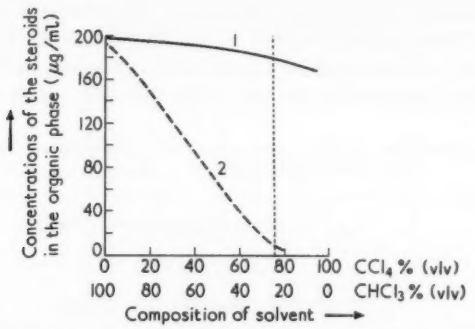

Fig. 3. Changes in the concentrations of 17-α-oxy-11-desoxy-corticosterone (1) and hydrocortisone (2) at different ratios of CCl_4 and $CHCl_3$ (in equilibrium with water)

nearly quantitatively, whilst hydrocortisone was extracted hardly at all. A single extraction with an equal volume of water suffices for the complete removal of the hydrocortisone from the organic phase. When applied to solutions of known concentrations of 17-α-oxy-11-desoxy-corticosterone, this method gave a linear calibration curve (Fig. 4). The 11-epi-hydrocortisone, which is more polar than the hydrocortisone, does not interfere; throughout the extraction it remains in the aqueous phase.

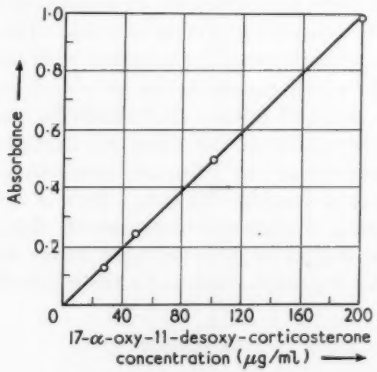

Fig. 4. Standard curve of 17-α-oxy-11-desoxy-corticosterone (for the assay of this substance in fermentation broths)

The conclusions drawn from the model experiments proved to be valid for fermentation liquors also. Experiments have been carried out with fermentation liquors containing no steroids; the results of these experiments are shown in Table II. The sulphuric

Table II. The absorbency of the chromogens in the fermentation broth without and with a known amount of steroid

A phase	50 ml of fermentation broth			
B phase	50 ml $CHCl_3$–CCl_4 (25 v : 75 v)			
Steroid, added	0	10 mg *a*	10 mg *b*	10 mg *a* 10 mg *b*
Absorbencies	0·005	0·852	0·042	0·897

a = 17-α-oxy-11-desoxy-corticosterone
b = hydrocortisone

acid chromogen produced in these experiments with fermentation liquors containing no steroids had no marked absorbancy at 5300 Å, the λ_{max}-value for the sulphuric acid chromogen of 17-α-oxy-11-desoxy-corticosterone. Thus the extinction attributable to the components of the fermentation liquor does not interfere with the determination; neither does the presence of hydrocortisone, as has been demonstrated in experiments with liquors containing added quantities of this steroid. The extinction of 17-α-oxy-11-desoxy-corticosterone together with the extinction of hydrocortisone in equal concentration is about the same as the extinction of 17-α-oxy-11-desoxy-corticosterone alone.

Applying this procedure to the microbial oxidation of 17-α-oxy-11-desoxy-corticosterone, the concentration values for this compound, plotted as a function of time, show a downward curve (Fig. 5) expressing the steady decrease of this concentration. From the actual degree of this decrease exact inference can be drawn concerning the stage reached by the oxidation process.

Procedure

From the fermentation broth originally containing 2 μg of 17-α-oxy-11-desoxy-corticosterone per millilitre, an aliquot (10·0

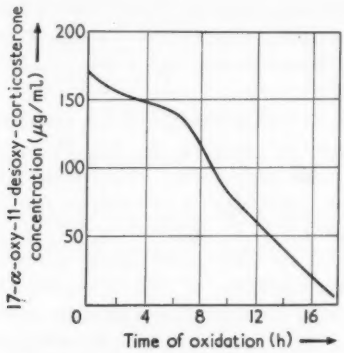

Fig. 5. Changes in the concentration of 17-α-oxy-11-desoxy-corticosterone during fermentative oxidation

ml) is transferred into a stoppered funnel. A 3 : 1 (v/v) mixture of carbon tetrachloride and chloroform (10·0 ml) is added, and the whole is vigorously shaken for 1 min. The organic phase is dried with one gram of anhydrous sodium sulphate. An aliquot

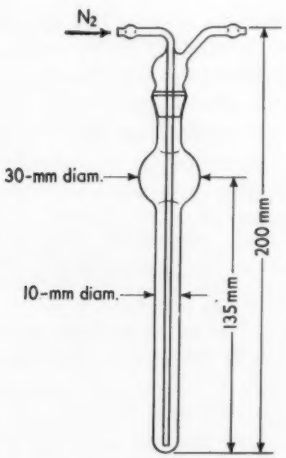

Fig. 6. Glass-vessel for the clarification of the sulphuric acid chromogen

(5·0 ml) of the organic phase is transferred to a glass-stoppered 100-ml Erlenmeyer flask, concentrated sulphuric acid (10·0 ml) is added, and the mixture shaken for 5 min. After this time, it is poured without delay into a separating funnel. After 2 min, the sulphuric acid phase is transferred to an aeration flask provided with a ground-glass fitting (Fig. 6), and there, to prevent their interfering with the photometric measurement, traces of the organic solvent are expelled with the help of a nitrogen gas stream (2 min, about 1 l./min). After standing for 5 min, the extinction value at $\lambda = 5300$ Å is determined spectrophotometrically. The corresponding concentration of the 17-α-oxy-11-desoxy-corticosterone is taken from a calibration curve previously plotted. In the preparation of the calibration curve, 10-ml portions of carbon tetrachloride–chloroform (3 : 1) solutions containing 200, 150, 100, 75, 50, 20, 10, and zero μg of 17-α-oxy-11-desoxy-corticosterone per millilitre are mixed intimately with 10-ml portions of a fermentation liquor containing no steroids. The procedure then follows the same route as described above, the extinctions being measured with the sulphuric acid chromogens of the pure solvent mixture as the blank. Extinctions were measured with a UNICAM S.P. 500 spectrophotometer.

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The Formation of Magnetic Iron Sulphide by Bacterial Reduction of Iron Solutions

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Summary. Laboratory scale experiments have been carried out with sulphate-reducing bacteria growing under anaerobic conditions to investigate the removal of iron from iron-containing solutions. The complete removal of iron, as the insoluble sulphide, has been achieved from both ferrous and ferrous–ferric sulphate solutions. It has been found that under certain conditions a magnetic iron sulphide is produced. This aggregates under the influence of a magnetic field and settles at a rate of some 30 in./min. The magnetic sulphide has only been obtained from intermittent continuous cultures. With the system employed, rates of removal of up to 150 mg of iron per litre of culture vessel per hour have been obtained.

Introduction

The conventional method for the removal of soluble iron involves neutralization with lime followed by sedimentation of the resultant hydrated iron oxides.[1] The main difficulty with this method is that of controlling the rate of precipitation so as to minimize the problem of de-watering.[2] It is known that certain micro-organisms, in particular members of the genus *Desulphovibrio*, can reduce sulphate to sulphide, and it was decided to investigate the possibility of using a microbiological system for the separation of iron from solution, as iron sulphide. These organisms are known to be present in humus tank sludge and this material was chosen as the inoculum source. In this paper, the experimental assembly used in the investigation is described in detail. A strictly practical approach to the problem was adopted and the results of experiments on the removal of iron from solutions of known constitution are reported and discussed.

Apparatus

A simple system was designed as illustrated in Fig. 1. A Pyrex desiccator was used as the culture vessel. The top was closed by a Perspex disc which was positioned by clamping plates secured to the cover of the Perspex water-bath. An endless piece of rubber tubing between the Perspex cover and the rim of the desiccator acted as a gas-tight gasket. Spare entries into the culture vessel were provided and when not in use were sealed by rubber bungs. Provision was also made for the passage of nitrogen through the gas space. The contents of the culture vessel were stirred by a

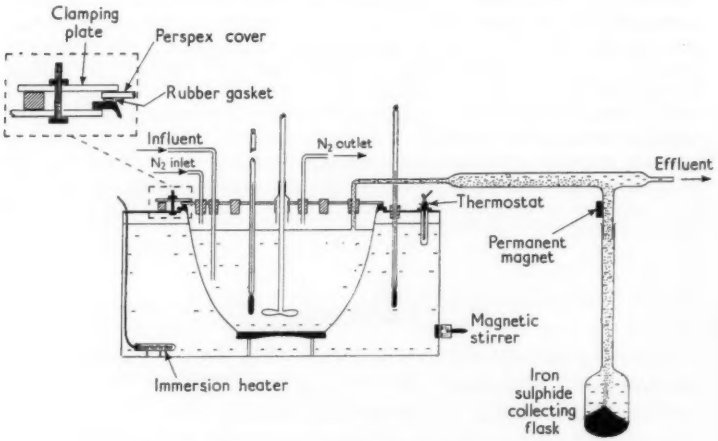

Fig. 1. Intermittent continuous culture apparatus

shafted variable-speed stirrer centrally situated and sealed by a flexible gas-tight gland. The water bath was heated by a 150-W immersion heater and its temperature was controlled by a bi-metallic strip thermostat (both of the type used in aquaria); and it was stirred by an external magnetic stirrer.

Influent was fed, from a reservoir, to the culture vessel through a positive displacement flow inducer. The vertical effluent take-off led to a wide bore horizontal tube with a T-junction connecting it to a flask in which the iron sulphide was collected. The

effluent, free of iron sulphide, passed to an effluent receiver which in turn was connected to a buffer flask and finally to a hydrogen sulphide scrubber containing 5 per cent cadmium acetate in 1 per cent acetic acid.

Inoculum

Humus tank sludge was used to inoculate the culture vessel. The sludge undoubtedly contained a mixed microbial population but as experimental runs proceeded there would probably be selective pressure towards the establishment of dominant organisms. It was thought that sulphate reduction, if it occurred, would probably be the result of the activity of *Desulphovibrio* strains and therefore environmental conditions of the culture were chosen to encourage the growth of these organisms. *Desulphovibrio desulphuricans* is reported to grow at pH values between 5 and 9, with the optimum pH range being 6·0 to 7·5.[3] The optimum temperature range is 25 to 30°C. The oxidation–reduction potential of the system is important[4, 5] and there is evidence that nitrates are inhibitory.[6–8] Cytochrome C_3[9, 10] is also important since a deficiency of it is associated with inability to reduce sulphate in *Desulphovibrio desulphuricans*. A critical review of sulphate-reducing bacteria is given by Postgate.[11]

Operating Conditions

Cultivation was carried out on an intermittent basis; i.e. the system was operated continuously during the day and left dormant overnight and at weekends. There was no recirculation of bacteria from the effluent to the culture vessel; the effluent was a culture and not a cell-free fluid. The system when in operation was, in fact, a single-stage stirred fermentor of the homogeneous open type.[12] Basically, in function it operated on the chemostat[13] principle.

The ferrous–ferric sulphate solutions were adjusted, finally to pH 6–6·5, and sodium lactate as the organic carbon source was then added. In the absence of ammonium ions, ammonium sulphate was added to provide a nitrogen source. When phosphate was not used for pH adjustment, a small amount was added as dipotassium hydrogen phosphate. The temperature of the

culture vessel was maintained at 30°C. The rate of removal of iron from solution, as iron sulphide, for a given retention time was controlled by the concentration of the organic carbon source. This concentration was adjusted to maintain a small residual of dissolved iron in the final effluent so preventing the production of free hydrogen sulphide. The influent was fed into the culture vessel at a constant rate of 10 ml/min which represented a retention time of 5 h for a culture vessel of 3 l. capacity. Stirring within the culture vessel was maintained between 400–500 rev/min. No provisions were made for sterilization or operation under aseptic conditions as pure cultures were not involved.

Determination of Iron Concentration

The ferrous iron remaining in solution in the final effluent was determined with total iron when ferrous–ferric solutions were employed. A standard method using 1:10 phenanthroline[14] was slightly modified for this purpose.

(*a*) *Total iron.* This was determined, as the ferrous phenanthroline complex, after reduction of the iron to the ferrous state. Effluent (10 ml), filtered free of suspended material, was placed in a 50-ml volumetric flask. To the flask were added 1 to 2 drops of concentrated hydrochloric acid, 5 ml of 10 per cent w/v hydroxylamine hydrochloride and a small piece of Congo red indicator paper. The contents were then titrated with 2 M sodium acetate solution until the indicator paper changed from blue to red (pH 5), and 5 ml of 0·25 per cent w/v 1:10 phenanthroline hydrochloride solution were added. The contents of the flask were made up to volume and allowed to stand for 20 min, and the optical density was measured in 1-cm matched cells at 5100 Å on a spectrophotometer against a blank of distilled water. Comparison with the calibration curve, which was linear and obeyed Beer's Law over the range 0–1·0 mg iron in a final volume of 50 ml, gave the concentration of total dissolved iron in the effluent.

(*b*) *Ferrous iron.* The dissolved ferrous iron in the effluent was determined by taking 10 ml of filtered sample into a 50-ml volumetric flask. To the contents of the flask were added 1 ml 2 M sodium acetate solution and 5 ml 0·25 per cent w/v 1:10 phenanthroline hydrochloride solution. The immediate appearance of a

red colour indicated the presence of ferrous iron. The contents of the flask were then made up to volume and the optical density measured as previously; again by reference to the calibration curve, the concentration of ferrous iron was determined.

Experimental Work

Experimental runs were conducted using FAS (ferrous ammonium sulphate); FAS and ferric sulphate; and ferrous and ferric sulphates mixed.

(*a*) *Ferrous ammonium sulphate* (*FAS*). The culturing system was initially started using FAS at a concentration of 425 p.p.m. Fe^{2+}, made up in de-oxygenated water, and 2·5 ml of a 70 per cent solution of sodium lactate were added per litre. The pH of the solution was then raised to 6·2 by the addition of a solution of 1 per cent di-potassium hydrogen phosphate. Three litres of the mixture were placed in the culture vessel and approximately 10 g of humus tank sludge added. The contents of the culture vessel were rapidly stirred for a few minutes to ensure initial distribution of the sludge after which stirring was discontinued. Nitrogen was slowly swept through the gas space passing from the apparatus via the hydrogen sulphide scrubber to the atmosphere. The constant-level effluent outlet remained closed. The system was then left in a static condition until there was evidence that iron sulphide was being produced; this was indicated by blackening on the interior walls of the culture vessel. First signs of blackening, in isolated spots, occurred after three days; slow stirring of the contents was then started and the system left for a further four days, by which time the whole of the contents of the culture vessel appeared black. After this period, the influent of FAS was fed into the culture vessel at a rate of 10 ml/min, the outlet opened and the stirring rate increased to about 1,000 rev/min. Subsequently the stirring rate was dropped to 400–500 rev/min. After two days' operation, the nitrogen inlet and outlet were closed and the anaerobic conditions within the culture vessel became self-sustaining.

During the initial trials, the iron sulphide produced tended to be dispersive and did not readily settle even after the addition of flocculants. After several weeks, it was assumed that the system

had attained a steady state. This was based on the fact that under the constant conditions employed the dissolved iron in the final effluent was virtually constant at 5 p.p.m. Fe^{2+}. This corresponded, approximately, to a rate of removal of 80 mg iron per litre of culture vessel per hour and approached an efficiency of 100 per cent. During the latter part of this period, it was observed that the iron sulphide was leaving the culture vessel in clusters of aggregated particles which settled rapidly under gravitational conditions. It was further noticed that the particles were strongly attracted towards a permanent magnet and that the entire material in suspension in the effluent appeared to be magnetic.* In this state the particles, if further aggregated under the influence of a magnetic field, subsequently settled under gravitational quiescent conditions at approximately three times the former rate. The system was maintained for seven weeks under the operating conditions described, during which time the physical properties of the resultant iron sulphide remained unaltered.

(*b*) *FAS and ferric sulphate.* The next experiment was conducted using FAS at a ferrous iron concentration of 300 p.p.m. Fe^{2+} plus ferric sulphate at a ferric iron concentration of 100 p.p.m. Fe^{3+}. Two millilitres of 70 per cent sodium lactate solution per litre were added, again to provide the organic carbon source, and the whole buffered with di-potassium hydrogen phosphate to pH 6–6·5. The influent flow rate was 10 ml/min. These conditions produced a highly magnetic material in the effluent, the residual iron in solution (wholly ferrous) varying between 15 and 20 p.p.m. Fe^{2+} and indicating that all the ferric iron had been removed or reduced to ferrous iron during the bacterial process. This rate of removal of iron corresponded to approximately 75 mg of iron per litre of culture vessel per hour. These conditions operated without change for two months.

(*c*) *Mixture of ferrous and ferric sulphates.* These experiments were conducted with a solution containing 700–800 p.p.m. total iron, of which 650–700 p.p.m. were ferrous iron. The solution was partially neutralized to pH 5·5 by the upward passage through a limestone bed, followed by final adjustment to pH 6·5 with sodium

* Magnetic iron sulphide is defined here as material which can be attracted by a permanent magnet, or material in which the individual particles are orientated under the influence of a magnetic field.

carbonate solution. Twenty p.p.m. of ammonia as ammonium sulphate (to provide a nitrogen source) and an organic carbon source of sodium lactate at a concentration of 4·0 ml of 70 per cent solution per litre were added. Under these conditions, a non-magnetic iron sulphide was produced which still settled rapidly leaving a clear effluent. The dissolved iron in the final effluent rose to between 40 and 50 p.p.m. Fe^{2+}. As a result the organic carbon source was increased to 5·0 ml of 70 per cent sodium lactate solution per litre and in addition 10 p.p.m. phosphate as dipotassium hydrogen phosphate were added. The system then reverted to its original condition, producing a highly magnetic material with a residual dissolved iron concentration in the final effluent of 1 p.p.m. The influent feed rate was 10 ml/min representing a rate of removal of some 150 mg of iron per litre of culture vessel per hour. The system has been operating satisfactorily for the past six months.

General Observations

In all the experimental runs, the rate of formation of magnetic material was controlled by the concentration of the organic carbon source and so adjusted that a residual of dissolved iron always remained in the final effluent. These conditions precluded the formation of free hydrogen sulphide which only occurred when all the iron present had been completely removed.

Some of the iron entering the culture vessel was in suspension, as the operating pH was consistent with the formation of some ferric hydroxide and ferrous hydroxide. The latter, when employing only ferrous iron solutions, in turn caused the formation of a small quantity of ferric hydroxide by oxidation, since although the influent reservoir was scoured with nitrogen no exacting precautions were taken to remove the last traces of dissolved oxygen present. However, the system converted iron present, whether in solution or in suspension, in the ferrous or ferric state, with apparent impartiality. Static cultures containing hydrated ferric oxides, ferrous hydroxide and mixtures thereof were converted to iron sulphide which was, however, non-magnetic.

The Magnetic Material

Magnetic material appeared to be produced only when culturing on an intermittent basis. Attempts to produce similar material

in stirred batch cultures under otherwise identical conditions failed. Chemical reproduction of the material, using chemically (and bacteriologically) produced hydrogen sulphide and passing it into various iron solutions with pH adjustment, likewise met with failure. The formation of magnetic material would therefore appear to be due to the physical conditions of culturing. The process is probably one of chemico-bacterial origin in which the controlling factors are a function of the bacteria and the physical operating conditions.

The percentage of water in the settled iron sulphide was roughly determined by weighing, centrifuging, decanting, washing and drying with alcohol and ether, and reweighing. The whole operation was carried out as rapidly as possible. The moisture content calculated on this basis was approximately 80 per cent.

Some of the magnetic material was oven dried at 110°C in an atmosphere of nitrogen and its density determined as 2·9, which is lower than the known values for sulphides of iron.[15] This, however, could be accounted for by the inclusion of cellular material from dead bacteria, or by the formation of partial hydrates stable or stabilized under the drying conditions employed.

The iron sulphide issuing from the culture vessel had an average settling rate of 10 in./min. If, however, settlement was preceded by aggregation of the particles under the influence of a magnetic field, the settling rate was increased to 30 in./min. The application of Stoke's Law to these settlement rates gave a particle size distribution of 50–110 μ diameter. This was supported by the fact that some of the material was retained on a 240 B.S. test sieve (60 μ) after passing through a 120 B.S. test sieve (120 μ).

The stability of the magnetic sulphide was examined under wet and dry conditions. Once dried under anaerobic conditions, the material appeared to be perfectly stable, retaining its magnetic properties even after repeated wetting and drying. Air drying produced apparent surface oxidation which retained magnetic material in the interior, exhibiting an overall decrease in magnetic properties but not eliminating them. Retention in water, without prior drying, under quiescent conditions open to the atmosphere, again produced superficial oxidation with the subsequent diminution of magnetic properties. Similar conditions but with continual agitation eventually oxidized all the material with complete

loss of magnetic properties. It would appear in the case of anaerobic drying that a passive film was produced on the surface of the material which precluded subsequent oxidation when exposed to the atmosphere or to dissolved oxygen in water.

The composition of the magnetic iron sulphide presented a difficult problem. Determination of the ferrous–ferric ratio (if any) by chemical analysis would have proved extremely difficult. In an attempt to establish its composition, some of the anaerobically dried material was subjected to an electron diffraction examination. Unfortunately an electron micrograph was unobtainable as the sulphide appeared to be amorphous in character. In the circumstances, a chemical analysis was attempted to determine the total iron and sulphur in the material. For the total iron determination, the material was taken into solution by prolonged boiling in 1:1 hydrochloric acid, reduced to the ferrous state with hydroxylamine hydrochloride and the iron determined as the 1:10 phenanthroline complex. The total sulphur content was determined by the high temperature combustion method used in conventional coal analysis.[16] Based on the analytical results, an empirical formula of Fe_4S_5 was established for the magnetic material examined.

Discussion

These preliminary experiments have demonstrated the removal of iron from solution by a microbiological process. The controlling factor in the process would appear to be the concentration of the organic carbon source. If the concentration of the organic carbon source is increased above the value for the stoichiometric requirements of the iron present, in relation to sulphur, then all the iron is removed and production of free hydrogen sulphide results. The ideal conditions are, therefore, to control the concentration of the organic carbon source at a value which permits a sufficient rate of bacterial action to remove the bulk of the iron, leaving a small residual of dissolved iron ($\nearrow 5$ p.p.m.) in the final effluent. This will then preclude the formation of free hydrogen sulphide.

No satisfactory explanation, at this stage, can be offered in respect of the formation and composition of the magnetic iron sulphide produced by the bacterial process.

Thorpe[17] describes a magnetic sulphide to which has been assigned the structural formula $5FeS.Fe_2S_3$ or $6FeS.Fe_2S_3$ corresponding to empirical formulae of Fe_7S_8 and Fe_8S_9 respectively. The iron sulphide produced by bacterial action could be assigned a structural formula of $2FeS.Fe_2S_3$. The ratios of Fe:S in all cases cited are apparently so similar that it might be argued that Fe_4S_5 is really identical with Fe_7S_8 or Fe_8S_9. Particularly in the latter case since Fe_4S_5 is equivalent to Fe_8S_{10} and would correspond in Fe:S ratio close to that for Fe_8S_9. However, from six separate analyses for total iron and sulphur (of the magnetic material) the iron combined with the determined sulphur was only 1·5 to 2 per cent higher than that demanded by the formula Fe_4S_5. whereas to correspond to a formula of Fe_7S_8 or Fe_8S_9, the iron value would need to have been at least 10 per cent greater than that actually found; this was considered to be outside the limits of experimental error. This is not intended to infer that the formula given by Thorpe is inaccurate, since it is feasible that both forms of magnetic iron sulphide can exist. That corresponding to Fe_7S_8 or Fe_8S_9 is of mineralogical origin whereas that of Fe_4S_5 is of bacterial–chemical origin, although there is no mention of the latter in the literature as far as is known.

The results of this work encourage the view that there may be a wide field in which the removal of metallic constituents from inorganic substrates by microbiological processes is both possible and applicable. This work is to be continued and the parameters involved in the process are to be studied in greater detail using six small culture units operating on a strictly continuous basis.

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The Recovery of Staphylococcal Enterotoxin from Bacterial Culture Supernatants by Ion Exchange*

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Summary. A method has been developed for the recovery of enterotoxin from bacterial culture supernatants of staphylococcus strain S-6 by use of the ion-exchange resin Amberlite XE–64 (IRC–50). The enterotoxin from 200 ml of bacterial culture supernatant diluted with one volume of water and adjusted to pH 6 can be removed with 1 g of resin pretreated with 0·02 M sodium phosphate, pH 6·2. The enterotoxin is recovered from the resin with 0·2 M sodium phosphate, pH 6·2, with subsequent precipitation with EtOH at $-10°C$, dialysis, and freeze drying. Partially purified preparations containing 1 emetic dose per 50μg were obtained.

Introduction

The etiological agent responsible for the most common type of food poisoning in the United States is staphylococcal enterotoxin. It is produced by certain strains of the staphylococci in foods or in simplified media in the laboratory. It is a protein of molecular weight 23,000 and has an isoelectric point near pH 8·5. Several methods have been used to recover the enterotoxin from bacterial culture supernatants, such as precipitation with $(NH_4)_2SO_4$, 40 per cent ethanol at $-7°C$, H_3PO_4 at pH 3·5, and zinc (Bergdoll, Sugiyama and Dack, 1959). This paper describes a method based on the removal of the enterotoxin from bacterial culture supernatants with the cation-exchange resin, Amberlite XE–64 (IRC–50).

Materials and Methods

Enterotoxin assay. The presence of enterotoxin was determined by the use of gel-diffusion techniques (simple and double diffusion) described previously (Bergdoll, Sugiyama and Dack,

* This work was supported by contributions from various companies and associations of the food industries.

1959, and Bergdoll, Surgalla and Dack, 1959). The simple diffusion technique as applied to enterotoxin involves the layering of an agar column containing enterotoxin antiserum with an enterotoxin solution. Under favourable conditions, the enterotoxin reacts with the anti-enterotoxin to form a band of precipitate in the agar–antiserum layer and the greater the concentration of the antigen the farther the precipitate band migrates into the agar. The double diffusion method involves the placing of a layer of agar containing buffer solution between the agar–anti-enterotoxin layer and the enterotoxin solution. The precipitate formed from the enterotoxin–anti-enterotoxin interaction appears in the agar layer as a sharp band, and the greater the concentration of the antigen the broader is the precipitate band and the nearer it is to the agar–anti-enterotoxin layer. The double diffusion method is more sensitive than the simple diffusion method. These methods have not been adapted for quantitative determination of the enterotoxin although equal quantities of enterotoxin in the same buffer solutions will give essentially equal migration of the precipitate band into the agar–anti-enterotoxin layer. Advantage was taken of this fact to compare the recoveries of enterotoxin from the resin in the different experiments. These methods were also used to determine the completeness of removal of enterotoxin from the bacterial culture supernatants by the ion-exchange treatment. They were not used to compare the amount of enterotoxin in the supernatants with that in the eluates because of the differences in effect of the solutions that contained the enterotoxin on the migration of the precipitate band.

The presence of enterotoxin was also determined by feeding enterotoxin solutions (usually 50 ml) to young rhesus monkeys (*Macaca mulatta*) by catheter (Bergdoll, Sugiyama and Dack, 1959, and Surgalla, Bergdoll and Dack, 1953). The animals were observed for 5 h after feeding, and vomiting was accepted as a positive reaction for enterotoxin. Usually each sample was fed to six monkeys and vomiting by at least two animals was considered evidence of the presence of enterotoxin. Although this method is only qualitative, it can be used to approximate the enterotoxin concentration of a given preparation. If two to four monkeys vomit following the administration of enterotoxin to unused animals, the conclusion is drawn that the amount of material

given contains at least one emetic dose. The results may vary from time to time by as much as 100 per cent.

Staphylococcus strain. Strain S–6 was used for the production of enterotoxin for these experiments.

Enterotoxin production. The enterotoxin was produced in an enzymehydrolyzed casein* medium as described elsewhere (Surgalla, Kadavy, Bergdoll and Dack, 1951). The bacteria were removed by centrifugation.

Amberlite XE–64 (IRC–50) resin. The finer resin particles were removed by mixing the resin with water several times and decanting each time after allowing the resin to settle for 30 min. The resin was treated with 500 ml of 40 per cent NaOH per lb of resin for 3 h with stirring. The alkali was decanted and the resin washed several times with water. The resin was treated with 9 l. of 3 N HCl per lb of resin over a 4-h period and then washed with water until the filtrate was chloride free.

Protein determinations. The relative concentrations of proteins and related substances were determined by the modification of the Folin method of Lowry *et al.* (1951).

Results

The procedures found to be satisfactory for the recovery of the enterotoxin from bacterial culture supernatants were as follows.

The XE–64 resin was placed in a chromatographic tube or other suitable container to a height of 4 cm, using 1 g of dry resin for each 200 ml of bacterial culture supernatant to be processed. The resin was washed with 0·02 M sodium phosphate solution, pH 6·2, until the percolate had a pH of 6·2.

The bacterial culture supernatant was diluted with one volume of distilled water and adjusted to pH 6 with H_3PO_4. The supernatant was passed through the resin bed by gravity although 2–3 lb pressure was helpful when passage became slow. Passage should be accomplished in 1–2 h. The resin bed was washed with 50 ml of 0·02 M sodium phosphate solution, pH 6·0, per gram of resin to remove any unadsorbed substances. The enterotoxin was removed from the resin with 80 ml of 0·2 M sodium phosphate solution, pH 6·2, per gram of resin.

* Obtained from Mead Johnson and Company, Evansville, Indiana.

The solution containing the enterotoxin was adjusted to pH 5 with H_3PO_4, cooled to 0°C and made up to 40 per cent alcohol with cold EtOH. The mixture was held at −10°C for 18–24 h before centrifuging. The precipitate was dissolved in distilled water and the pH adjusted to 7·5. The solution was then dialyzed against distilled water, centrifuged to remove any insoluble material, and freeze-dried.

The freeze-dried material contained 12–15 per cent nitrogen and 50 μg of it caused emesis in two to four monkeys of a group of six.

Discussion

The ion-exchange resin XE–64 (IRC–50) has been used previously in the purification of enterotoxin (Bergdoll, Sugiyama and

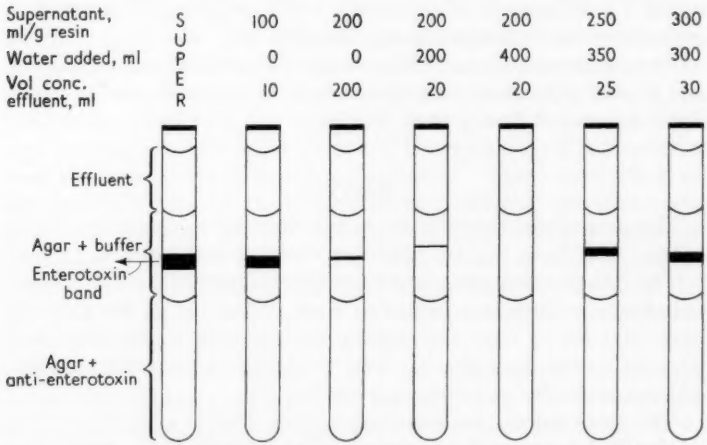

Fig. 1. Enterotoxin remaining in effluents after passage of bacterial culture supernatants through XE–64 resin as determined by double diffusion

Dack, 1959). Partially purified enterotoxin was adsorbed by this resin from 0·02 M sodium phosphate solution, pH 6·2, with subsequent recovery from the resin with 0·2 M sodium phosphate solution, pH 6·2. Attempts to recover the enterotoxin from bacterial culture supernatants using the same conditions were completely successful only after dilution of the supernatants with

water. Apparently the better adsorption of the enterotoxin from the diluted supernatant was due to the lower ionic strength. Results given in Fig. 1 indicate that adsorption was complete when two volumes of water were added, but figures in Table I

Table I. Enterotoxin remaining in effluents after passage of bacterial culture supernatants through XE–64 resin, as determined by simple diffusion

Bacterial supernatant, ml/g resin	H_2O added, ml	Volume of concentrated effluent, ml	Enterotoxin band migration,[a] mm
100	0	10	7·0
200	0	200	7·0
200	200	20	0
200	400	20	0
250	350	25	0
300	300	30	9·0
Untreated bacterial supernatant		unconc.	9·0

[a] The enterotoxin concentration is roughly proportional to the band migration.

show that the amount not adsorbed when one volume of water was used was negligible. When the effluents from the passage of the supernatants through the resin were tested for enterotoxin, they were freeze-dried and then dissolved in water to give the desired concentration. The maximum amount of supernatant per gram of resin appeared to be approximately 200 ml.

The eluates from the XE–64 resin in many of the experiments in this investigation were collected in 2-ml fractions with the aid of a fraction collector. Determinations were made on 0·1-ml aliquots of each fraction to determine the relative amounts of proteins and related substances adsorbed by the resin and their distribution in the eluate. Normally the enterotoxin was not confined to one part of the eluate, but was distributed throughout it. Eighty millilitres of 0·2 M phosphate solution, pH 6·2, per gram of resin was more than sufficient to remove the enterotoxin.

The nature and quantity of products produced by the staphylococci may influence the adsorption of the enterotoxin. A study of culture supernatants obtained by inoculation with 100 different single colony isolates from the staphylococcus strain, S–6, showed a wide variation in the amount of enterotoxin produced as well as the amount of other materials adsorbed by the XE–64 resin.

Fig. 2 shows typical variations in the resin-adsorbable substances produced by the staphylococci. Large amounts of resin-adsorbable substances may be produced by staphylococci that produce very little enterotoxin and vice versa. The smaller the total amount of adsorbable substances in the supernatant the larger the amount of enterotoxin that was adsorbed by the resin (Fig. 3). Supernatant 2, which contained 4–5 times as much enterotoxin as supernatant 1, but less total adsorbable substances, could be

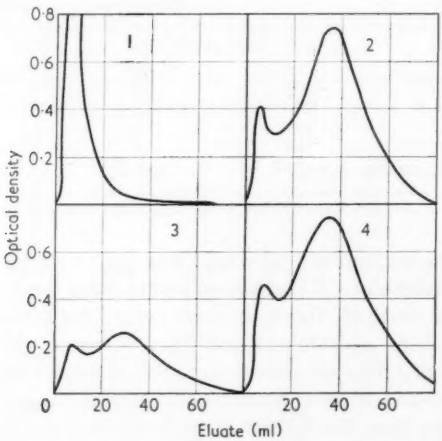

Fig. 2. Proteins (including enterotoxin) and related substances adsorbed by XE–64 resin from bacterial culture supernatants from selected single colony isolates of staphylococcus strain S–6 when 100 ml of the supernatant was diluted to 300 ml (pH 6) and passed through 1 g of resin pretreated with 0·02 M sodium phosphate solution, pH 6·2, and eluted from the resin with 0·2 M sodium phosphate solution, pH 6·2. Eluates were collected in 2-ml fractions and substances present in the fractions determined in 0·1-ml aliquots by the Lowry method. Enterotoxin band migration at 7 days with untreated supernatants: 1, 16·4 mm; 2, 0 mm; 3, 18 mm; 4, 23 mm

used in equal quantity per gram of resin. This problem may be simplified by pre-selection of a colony that produces a large amount of enterotoxin, but a small amount of total adsorbable substances.

The media used for production of the enterotoxin may influence the adsorption of the enterotoxin if large amounts of adsorbable

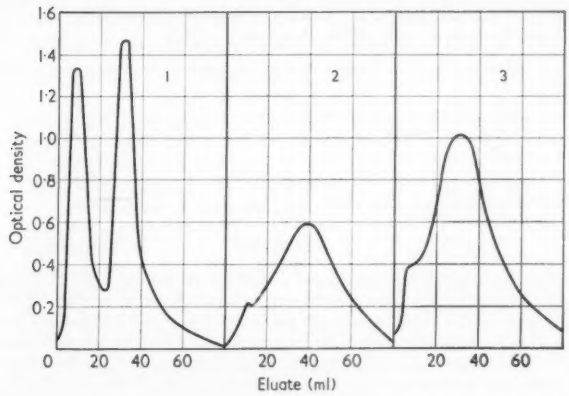

Fig. 3. Proteins (including enterotoxin) and related substances adsorbed by XE–64 resin from bacterial culture supernatants when 200 ml were diluted to 600 ml (pH 6) and passed through 1 g of resin pretreated with 0·02 M sodium phosphate solution, pH 6·2, and eluted from the resin with 0·2 M sodium phosphate solution, pH 6·2. Eluates were collected in 2-ml fractions and substances present in fractions were determined in 0·1-ml aliquots by the Lowry method. Enterotoxin band migration at 7 days with untreated supernatants: 1, 9 mm with 1 × conc.; 2, 10·2 mm with 0·25 × conc.; 3, 11·5 mm with 0·125 × conc. Enterotoxin band migration at 7 days with effluents from passage of supernatants through resin: 1, 0 mm with 10 × conc; 2, 0 mm with 10 × conc; 3, 3·9 mm with 1 × conc. When larger amounts of supernatant 1 were used, enterotoxin appeared in the effluent

substances are present. In this investigation, the enzyme-hydrolyzed casein used in the production of the enterotoxin contained only small amounts of adsorbable substances (Fig. 4).

The depth of the bed of resin is not of particular importance except that too shallow beds may be difficult to maintain at uniform depth and beds too deep may hinder the flow of liquid through them.

Most of the experiments in this investigation were done with resin conditioned with 0·02 M sodium phosphate solution, pH 6·2. A few experiments with water-washed resin indicated that more enterotoxin was adsorbed when this treatment was used than with the phosphate treated resin (Table II). However, much more gas was evolved during the passage of the supernatants through the water-washed resin. It is not known that this adversely affected

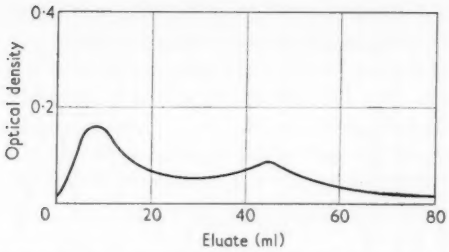

Fig. 4. Proteins and related substances adsorbed by XE–64 resin from 2 per cent enzyme-hydrolyzed casein (Mead Johnson) when 100 ml were diluted to 300 ml (pH 6) and passed through 1 g of resin pretreated with 0·02 M sodium phosphate solution, pH 6·2, and eluted from the resin with 0·2 M sodium phosphate solution, pH 6·2. The eluate was collected in 2-ml fractions and substances in the fractions determined in 0·1-ml aliquots by the Lowry method

Table II. Enterotoxin remaining in effluents after passage of bacterial culture supernatants through XE–64 resin, as determined by simple diffusion

Supernatant, ml/g resin	H_2O added, ml	XE–64 Resin Treatment	
		H_2O, mm[a]	0·02 M Na phos., pH 6·2, mm[a]
100	0	0	8·5
200	0	9·7	15·0
200	200	0	0

[a] Enterotoxin band migration with effluents concentrated ten-fold.

the enterotoxin, but buffering the resin before adsorption should make any damage to the enterotoxin less likely.

Neither of the two methods for the detection of enterotoxin have been used quantitatively to determine the percentage recovery of the enterotoxin. When attempts have been made to compare the enterotoxin in partially purified preparations with that in bacterial culture supernatants, there appears to have been much less enterotoxin in the partially purified materials regardless of the method of preparation. Normally, if the partially purified preparations contained 50 per cent of the activity found in the supernatants, recovery was considered satisfactory. Typical results of the recovery of enterotoxin by the ion-exchange method as evidenced by the monkey feeding test are given in Table III.

The gel-diffusion test was used to compare the recoveries of enterotoxin in the resin eluates within a series of experiments. Results of these comparisons are given in Table IV. To make

Table III. Comparison of enterotoxin in bacterial culture supernatant and partially purified preparations by monkey feeding test

Enterotoxin sample	Amount fed, ml	Feeding results, monkeys vomited/monkeys fed
Supernatant 1	0·125	3/6
Resin eluate 1	0·250[a]	5/12
Supernatant 2	1	3/6
Resin eluate 2 (alcohol precipitate)	2[a]	4/6

[a] Equivalent volume of bacterial culture supernatant.

these comparisons, the eluates containing the enterotoxin were adjusted so that the concentrations of enterotoxin per millilitre of the eluate would be equal to each other if the enterotoxin recovered was proportional to the amount of supernatant used.

Table IV. Enterotoxin in eluates from XE–64 resin as determined by simple diffusion

Bacterial culture supernatant, ml/g resin	XE–64 resin treatment			
	H_2O		0·02 M Na phosphate, pH 6·2	
	Supernatant undiluted, mm[a]	Supernatant + 1 vol H_2O, mm[a]	Supernatant undiluted, mm[a]	Supernatant + 1 vol H_2O, mm[a]
10	—	6·8	—	7·0
20	—	6·7	—	7·5
50	8·1	7·8	8·4	8·1
100	8·4	7·9	8·4	8·1
200	7·1	8·2	7·7	8·1

[a] Enterotoxin band migration at 8 days from eluates diluted proportionately to amount of supernatant used.

The enterotoxin preparations obtained by the ion-exchange method were similar in potency on a dry-weight basis (50 μg per emetic dose) to those previously prepared from bacterial culture supernatants by acid precipitation, adsorption on alumina, and alcohol precipitation (Bergdoll, Sugiyama and Dack, 1959).

Although the total amount of enterotoxin obtained depended on the potency of the supernatants, in general it appeared that the amount of enterotoxin recovered by the ion-exchange method was about twice that obtained by the previous method. The ion-exchange method is simpler in that it replaces both the acid precipitation and alumina adsorption steps. The resin is easier to condition with buffer than the alumina and is a milder adsorbent, hence less likely to damage the enterotoxin. There appears to be no limitation to the quantity of bacterial culture supernatant that can be processed.

The ion-exchange method has been used primarily with enterotoxin produced by strain S–6. Preliminary experiments indicate that it can be used to recover the enterotoxin in bacterial culture supernatants from other strains; however, the smaller amount of enterotoxin produced by these strains may necessitate some alteration in the conditions used.

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Production of Polysaccharide with *Xanthomonas campestris*

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Summary. Industrially important polysaccharides are now primarily obtained from plant sources. One exception is the exocellular bacterial polysaccharide, dextran. A survey at the Northern Laboratory revealed that many other microbial polysaccharides could be synthesized by the action of micro-organisms on starch-derived saccharides. Previous publications from this laboratory describe the production of the microbial polymer, phosphomannan, synthesized by the yeast *Hansenula holstii*. This paper describes the production of another polysaccharide, synthesized by the bacterium *Xanthomonas campestris* NRRL B–1459.

Fermentation of media containing 3 per cent dextrose, seeded with 5 per cent inoculum of *X. campestris* NRRL B–1459 and cultured aerobically at 28°C, is complete in 96 h. A light tan polymer is recovered in yields of 50 per cent, based on commercial dextrose, from the viscous fermentation broth by precipitation with methanol in the presence of an electrolyte. The viscosities of aqueous re-solutions containing 1 and 2 per cent of the polymer were 3,000 cP and 11,000 cP respectively.

Introduction

Polysaccharides, which currently are used industrially, are obtained chiefly from plant sources. These materials include cellulose, starch, pectins and other plant gums, such as seaweed extractives, tree exudates and those associated with seeds. An important exception is the exocellular bacterial polysaccharide, dextran, which has been produced for industrial application as well as for use as a blood–plasma volume expander.[1–3] Many studies of other microbial polysaccharides have been made from the standpoint of their immunological properties, but there have been few reports on the production of microbial polysaccharides

* This is a laboratory of the Northern Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture.

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by mass culture techniques. Two papers from this laboratory described a process developed for the production of a phosphorylated mannan by the yeast *Hansenula holstii*.[4, 5] Lilly *et al.*[6] investigated processes for the laboratory production of exocellular polysaccharides by species of *Xanthomonas*. Although potentialities for polysaccharide production by micro-organisms have been noted, there has been little investigation of microbial gums from the standpoint of industrial manufacture.[7]

The work reported here summarizes the research undertaken to develop a process for the production of polysaccharide by the bacterium *Xanthomonas campestris* NRRL B–1459.

Experimental

Equipment

Fermentation studies were conducted in fermentors that ranged in size from laboratory-shaken flasks to a 600-gal vessel. In the laboratory-scale investigations, 300-ml Erlenmeyer flasks containing 75 ml of medium were used for a major portion of the work. Tests were also conducted with 2,800-ml Fernbach flasks in which the culture volume was 800 ml. Flasks were incubated at 25°C either on a rotary shaker at 200 rev/min ($2\frac{1}{2}$ in. eccentricity) or on a reciprocal shaker having an amplitude of $2\frac{1}{4}$ in. and a frequency of 100 strokes/min. In the 20-l. fermentors, previously described by Dworschack *et al.*,[8] 10 l. of medium was used. Pilot-plant fermentations were conducted in two stainless-steel vessels of 60- and 600-gal capacity containing either 20 or 30 gal of medium in the smaller and 200 gal in the larger vessel.

The 60-gal fermentor was 21 in. in diameter, 48 in. high, and was jacketed. Agitation was provided by a 3-blade, 8-in. propeller mounted at the bottom of a top-entering shaft and powered by a $\frac{1}{3}$-h.p. motor. Agitator speed varied from 90 to 290 rev/min. Sterile air was introduced through a sparger directly below the agitator.

The 600-gal fermentor was 48 in. in diameter, approximately 90 in. high, and was jacketed. Its contents were agitated with a top-entering, flat-blade, 18-in. disk turbine that had eight 3-in. blades and was driven by a 3-h.p. motor. Agitator speed varied

from 50 to 250 rev/min. Sterile air was introduced through a sparger directly below the disk turbine.

Air was sterilized by passage through a 12-in. column packed with 6 ft of 10- to 24-mesh carbon. Automatic controls maintained temperatures during fermentation within 1° of the desired temperature, 28°C.

The medium was sterilized continuously with a steam jet heater and a 3-in. stainless-steel holding coil as a retention chamber.

Cells were removed from the fermented media by centrifuging with a No. 16 Sharples Supercentrifuge.*

Precipitation and purification were performed in type 304 stainless-steel tanks, equipped with propeller-type air-driven agitators.

A dry product was recovered by dehydrating with methanol and drying in either a vacuum tray or vacuum rotary drier.

Microbiology

Xanthomonas campestris NRRL B–1459 was obtained from the ARS Culture Collection where it is maintained as a lyophilized stock culture. Viable cultures for inoculum build-up were maintained on malt extract–yeast extract (MY) agar slants and were transferred weekly to minimize lag in the inoculum phase.[9] Freshly inoculated slants were incubated for 1 or 2 days at 25°–28°C, then refrigerated for routine use. In the preparation of inoculum, growth from a slant was transferred to MY broth (100 ml in a 500-ml Erlenmeyer flask); incubated 24 h on the reciprocating shaker at 25°C; and used (10 per cent v/v) to seed the inoculum flasks. The medium for inoculum production contained commercial corn sugar, 30·0 g/l.; distillers' dried solubles, 4·0 g/l.; dipotassium hydrogen phosphate, 5·0 g/l.; and magnesium sulphate heptohydrate, 0·1 g/l. Inoculum flasks were incubated for 24 h on the reciprocating shaker at 25°C, and the cultures were then used to inoculate fermentations for the production of polymer. When large amounts of inoculum were required, the 60-gal fermentor was used as a seed tank. In all instances, 5 per cent (v/v) inoculum was used to inoculate fermentations.

* Sharples Corp., 2362 Westmoreland, Philadelphia 40, Pennsylvania.

Fermentation

Shaken flasks were sterilized in a standard laboratory autoclave at 121°C for 15 min. Sterilization of the 20-l. fermentors was accomplished by injecting steam into both the jacket and the medium and holding the temperature at 121°C for 5 min. To avoid charring of the medium, the glucose solution was sterilized separately in the fermentors and the remainder of the medium was pre-sterilized and added aseptically to the sterile fermentors. In pilot-plant fermentations, the medium was sterilized continuously. All equipment was steam sterilized at 121°–126°C for 2 h before use; the medium ingredients, as stated for inoculum production, were added to water in a mixing tank; the pH was adjusted to 7·0; and the medium was then pumped at a constant rate to a steam jet heater where it was mixed with steam to 135°C instantaneously. After 4 min retention in a holding coil, the medium in its continuous passage to the fermentor was cooled in a spiral heat exchanger to the required fermentation temperature, 28°C. After the fermentor was inoculated, agitation and aeration were adjusted so that the oxygen-absorption rate was 1·0 mmoles of oxygen/l./min when measured according to the sulphite method of Cooper, Fernstrom and Miller.[10] The progress of the fermentation was followed by determining the pH, the sugar concentration, the viscosity and the yield of polymer.

The medium composition for the larger scale fermentations was chosen on the basis of experiments conducted with shaken flasks. The objective was to find a practical, economical medium that would support production of good yields of polymer. Experiments were made to study the effects of pH, glucose concentration, and type and concentration of organic nitrogen sources.

Recovery of Polysaccharide

In reporting how polysaccharide B–1459 was recovered, all methanol concentrations are expressed as per cent by weight. Cells and suspended impurities were removed from the fermented broth by centrifugation after adjusting the pH to 5·6 and decreasing the viscosity below 700 cP with 24·7 per cent methanol. A soft gel-like crude polymer was precipitated from the centrifugate after adding 2 per cent KCl and additional methanol to 50

per cent concentration. The precipitate was recovered by centrifuging. The crude polysaccharide was purified by re-precipitating the polymer from an aqueous solution containing 1 per cent polymer by adding 2 per cent KCl and methanol to 50 per cent concentration. The polymer was decolorized and dehydrated by suspending the gum in four parts of methanol and filtering. This step was repeated twice. Residual methanol was removed by vacuum drying, and the dry polymer was ground in a hammer mill.

Analytical Methods

Reducing sugar concentration in the culture medium was determined by the Somogyi or Shaffer and Hartmann methods.[11, 12] Under the conditions of analysis used, the polysaccharide in the culture fluid did not exhibit reducing power.

Polymer content of the culture liquor was determined as follows:

A weighed aliquot of culture was diluted to reduce viscosity and centrifuged to remove bacterial cells and other suspended solids. To 10 ml of the resulting supernatant liquid, 3 drops of saturated KCl and 20 ml (2 vol) of 95 per cent ethanol were added. The mixture was vigorously shaken to break up the gummy precipitate as it formed. After centrifugation, the precipitate was washed with ethanol and partially dehydrated by washing with acetone several times, and then dried to constant weight in an air or vacuum oven. Samples were usually dry in about 6 h. For pilot-plant work, a rapid method was developed which gave results within 5 per cent of the laboratory method: 2 g of KCl was dissolved in 100 g of broth and 190 ml of methanol was added with agitation. The suspension was filtered through a 6-in. stainless-steel 60-mesh funnel and after draining for 30 min the wet precipitate was weighed. Dry product was calculated by using the factor 0·02.

Polymer production during fermentation was also estimated by determining the viscosity of the whole culture. In the small-scale fermentations (shaken flasks and 20-l. fermentors), 'flow-time' of samples was measured. Flow-time can be defined as the number of seconds required for a sample to be delivered from a standard 4-ml volumetric pipette. Flow-times were proportional to polymer concentration only at relatively low polymer levels;

hence, these data were used only as indications of conversion rates. When larger samples were available, as was the case with the 60- and 600-gal fermentors, viscosity was determined at 30 rev/min and 25°C with a Brookfield Model LVT Viscometer.*

Results and Discussion

A process for producing a polysaccharide with *Xanthomonas campestris* NRRL B–1459 was developed sequentially from laboratory-shaker flasks and 20-l. fermentors to 60-gal and 600-gal fermentors. Because separation and purification of the water-soluble polysaccharide are impeded by residual raw materials in the fermented culture liquor, efforts were made to obtain nearly complete utilization of the carbohydrate and to have present only the quantity of nitrogen source necessary for optimum polymer production. Preliminary experiments in shaken flasks with various complex nitrogen sources, such as meat by-products and yeast preparations, indicated that several substances were satisfactory for growth of the organism. Among the proteinaceous materials tested, distillers' dried solubles afforded the best yields of polysaccharide. Further studies with various concentrations of distillers' dried solubles showed that 0·4 per cent was the lowest level that would support maximal formation of polymer under our fermentation conditions.

Results of experiments to determine the optimal concentration of glucose are shown in Fig. 1. When 1 per cent glucose was used, conversion was nearly quantitative, and 90 per cent of the glucose was recovered as polysaccharide. However, as the glucose concentration was increased, the fermentation efficiency, i.e. yield of polymer calculated on initial glucose concentration of the medium, decreased markedly. This effect was also observed in the change in polymer concentration of the culture liquor. As the sugar level was raised to about 4 per cent, the polymer concentration increased and then remained nearly constant regardless of the amount of glucose available for fermentation. Approximately 2·5 g of glucose per 100 ml of medium was consumed regardless of the initial concentration. Hence, it appears that 2·5 to 3 per cent glucose is optimal.

* Brookfield Engineering Laboratories, Stoughton, Massachusetts.

Efforts to increase polymer concentration and utilization of glucose by starting the fermentation with only 0·5 to 1·0 per cent glucose, then intermittently adding sterile glucose during incubation, resulted in the same ultimate yield of polysaccharide and the same net utilization of glucose as obtained when all the glucose was added at the beginning of the fermentation.

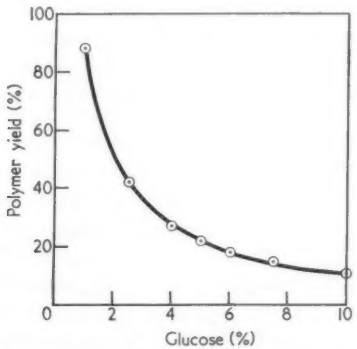

Fig. 1. Effect of initial glucose concentration on polymer yield

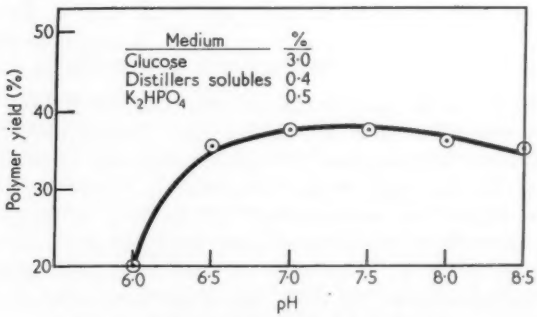

Fig. 2. Effect of initial pH on polymer yield

Experiments were run to ascertain the most desirable pH for polysaccharide production. Initial pH values were varied from 6·0 to 8·5 at 0·5 pH intervals. The results of the 4-day fermentation are shown graphically in Fig. 2. There is a broad optimum

centring about pH 7·2. Yields decrease more rapidly at slightly acid pH values than they do at slightly alkaline levels. Since the fermentation is slightly acidogenic, the pH remains in the optimum range longer when initiated at somewhat alkaline pH; conversely, when slightly acid pH is used, the acidity produced in the culture fluid drops the pH below the optimum value more rapidly.

The course of a typical shaken-flask fermentation is presented graphically in Fig. 3. Fernbach flasks were incubated on a rotary shaker as described and samples were withdrawn for analyses daily. Each point on the graph represents the average

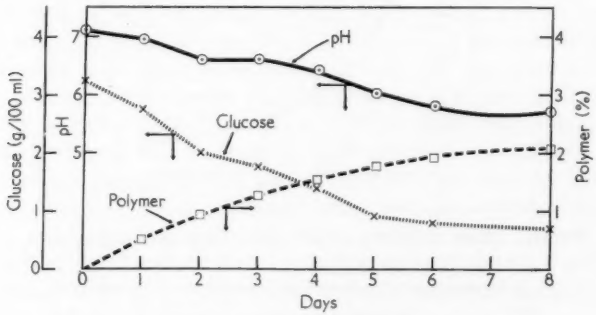

Fig. 3. Changes during fermentation in shaken flasks

of determinations on samples from three different flasks. Yield data were calculated from the initial sugar concentration. The rate of glucose consumption decreased markedly after 4 days' incubation; at this stage of the fermentation nearly one-third of the initial sugar was unfermented. There was no appreciable further utilization of glucose even when the incubation period was extended to 8 days. The rate of polysaccharide production steadily decreased after the third day of the fermentation period; hence, there was little advantage gained from the standpoint of polymer yield by prolonging the incubation beyond 5 or 6 days. Changes in pH in the illustrated shaken-flask experiments are typical of those obtained in fermentations on a larger scale; *viz.* there is a steady, gradual decline from the initial level, 7·1, to the final value of about 5·8 to 6·2.

Preparatory to large-scale fermentations, studies were made in 20-l. fermentors to establish suitable levels of aeration and agitation. Investigation of agitation rates in baffled fermentors showed that excessive agitator speeds were detrimental when used in the early stages of the fermentation. After 36 to 48 h incubation, viscosity of the culture liquid had increased sufficiently to protect the bacterial cells from the injurious effects of violent agitation and the stirring rate could then be increased. Best yields and most rapid development of viscosity were obtained when the fermentations were agitated at 150 rev/min for the first 48 h, then at 280 rev/min for the remainder of the incubation period. An aeration rate of 0·5 v/v per min was suitable; lower rates caused slower fermentations, higher air rates were not beneficial. The fermentor head pressure was maintained at 15 lb/in^2. The aeration rate and pressures employed gave oxygen absorption rates ranging from about 0·4 mmoles oxygen/l./min at the lower agitator speed to 1·6 mmoles at the higher rate.[10] These values are not indicative of dissolved oxygen concentration in the actual fluid because of the extreme viscosity of the polysaccharide solution, particularly during later stages of the fermentation. The course of typical experiments with the 20-l. fermentations is very similar to that shown for the 60-gal fermentor in Fig. 4. Data from these fermentations are summarized in Table I. The experimental conditions employed in the 20-l. fermentations were suitable for polymer production in the larger pilot-plant facilities.

Pilot-plant fermentations were carried out primarily to produce polysaccharide B–1459 for industrial evaluation; consequently, variables were not studied, and fermentations were conducted using the best conditions as developed in the laboratory for shaken flasks and 20-l. fermentors. Comparable results were obtained in the first 60 h for both the 60- and 600-gal fermentors. After 60 h, when the viscosity of the broth was *ca.* 3,000 cP, metabolic changes were much slower in the 60-gal fermentor. Fermentation was complete in 118 h compared to 94 h for the 600-gal vessel. This is attributed to a decrease in oxygen absorption in the smaller fermentor because of insufficient agitation as the broth became more viscous. Although final viscosities were about the same, the yield of polysaccharide was slightly higher in the larger

fermentor. Chemical and physical changes during fermentation in both fermentors are shown in Fig. 4.

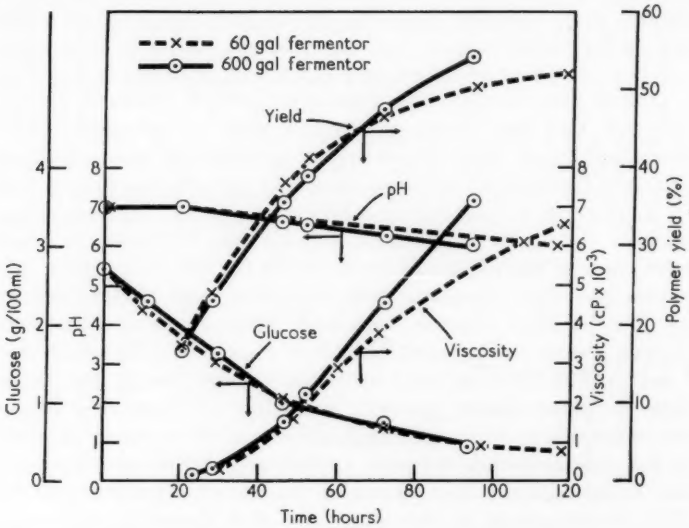

Fig. 4. Changes during fermentation in pilot-plant fermentors

Table I. NRRL B–1459 Polysaccharide production summary data

Fermentor capacity	Medium volume	Initial glucose, %	Fermentation time, h	Average yield[a]		Polymer concentration, %
				Calcd.	Recovered	
2800 ml	800 ml	2·7	144	62·0	—	1·86
20 l.	10 l.	4·5	120	34·0	—	1·75
60 gal	20 gal	4·5	118	—	16·9	—
60 gal	20 gal	2·7	118	50·0	42·7	1·50
60 gal	30 gal	4·5	118	—	19·0	—
60 gal	30 gal	2·7	118	51·2	41·4	1·53
600 gal	200 gal	2·7	94	54·0	50·6	1·62

[a] Pounds of polymer/100 lb of commercial dextrose added to fermentor.

Recovery of B–1459

Because of the high viscosity, *ca.* 7,000 cP, of the fermented broth, it was difficult to remove cells. However, tests showed that most of the cells were removed by one centrifugation when the viscosity was below 700 cP; therefore, the broth was diluted by adding an equal weight of water and methanol to 24·7 per cent concentration. Diluting with methanol effectively decreased the viscosity without precipitating polymer. If a vapour-tight centrifuge is available, a higher concentration of methanol can be used and less water is required, thereby decreasing the overall quantities of methanol needed for subsequent polysaccharide precipitation.

The polymer does not precipitate from solution with methanol unless an electrolyte such as KCl is present. A soft gel-like precipitate formed in the cell-free liquor after adding 2 per cent KCl and additional methanol to 50 per cent concentration. The precipitate was recovered by centrifuging and a tan-grey fibrous polymer was obtained which contained 0·42 per cent nitrogen and 29·8 per cent ash. The crude polysaccharide was purified by dissolving it to 1 per cent concentration with distilled water, adding 2 per cent KCl, adding methanol to 50 per cent concentration, and centrifuging the slurry. The recovered precipitate was partially decolorized and dehydrated by suspending the polymer in four parts of methanol and filtering. This step was repeated twice. The polysaccharide was vacuum-dried at 28 in. Hg and 104°F to remove residual methanol, and ground in a hammer mill. The resulting light-tan powder contained 0·33 per cent nitrogen and 11·2 per cent ash. The yield of polysaccharide recovered was *ca.* 50 per cent based on commercial dextrose added to the fermentor. When city water was substituted for distilled water in the processing, the colour of the polymer was grey rather than tan. It contained 0·35 per cent nitrogen and 13·4 per cent ash. The viscosities of solutions of both products were similar (Fig. 5).

Methanol used in the dehydration and decolorization was recovered and re-used in the precipitation step. Supernatants from precipitation were redistilled to recover methanol.

Because *Xanthomonas campestris* is a plant pathogen, the centrifuged cells were killed by steam heat before discarding.

Other precipitation and recovery methods are now being investigated in the pilot plant.

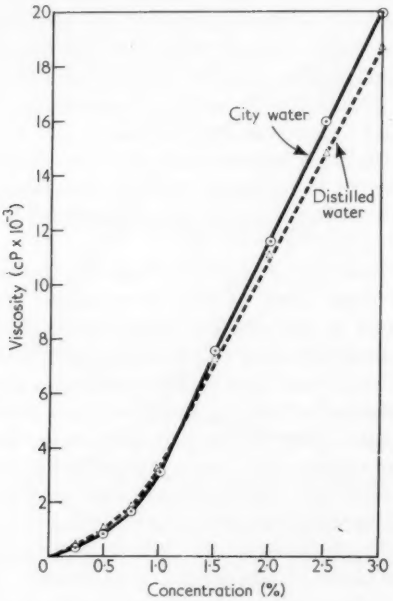

Fig. 5. Kind of water used in recovery of polysaccharide did not affect viscosity

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Spray-drying of Tomato Puree, Groundnut and Soyabean Proteins and Sugar–Gelatine Solutions

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Summary. Tomato puree, groundnut and soya protein, and sugar–gelatine solutions were successfully dried in a Scott pilot plant spray-drier. The tomato puree did not need the addition of a carrier provided that the air plenum of the drier was suitably modified. Spray-drying did not change the colour of tomato puree and the protein solutions, the viscosity characteristics of the protein solutions, or the nutritive value of the proteins. The conditions for the successful spray-drying of sugar–gelatine solutions were rather critical. The temperature in the spray-drier had the major effect on the quality of the powders. Published data on spray-drying do not provide a basis for predicting the drying behaviour of different products; experiments on the plant are essential.

Introduction

Seltzer and Settlemeyer[1] describe various commercial types of spray-driers and Friedman *et al.*[2] have described work on centrifugal disc atomization. The factors required for the selection and design of a spray-drier have been described by Laster,[3] and Marshall[4] has discussed the principles of spray-drying. Current theories on atomization by high pressure and two-fluid type nozzles, and centrifugal discs are discussed by Fraser *et al.*[5] Patents on improvements and modifications of spray-driers have frequently appeared, e.g. Campbell[6] has arranged air flow through tangential adjustable valves to provide maximum efficiency. There are numerous references to the spray-drying of particular food products, e.g. eggs,[7] milk,[8] wheat gluten,[9] cream,[10] chocolate products,[11] potatoes,[12] and tomatoes.[13]

We have studied the special applications of spray-drying, rather than the fundamentals, on a Scott pilot plant spray-drier, of evaporative capacity 40 lb water per hour for milk containing 40 per cent solids. Fig. 1 shows a schematic diagram of the co-current pilot plant spray-drier using a spinning disc atomizer.

Tomato puree, groundnut and soyabean protein, sugar–gelatine mix and high fat emulsions were successfully dried only after modifying the pilot plant drier. Fish protein and glyceryl monostearate–milk mixture were not amenable to spray-drying. The drying of tomato puree, vegetable protein and sugar–gelatine is described in detail below.

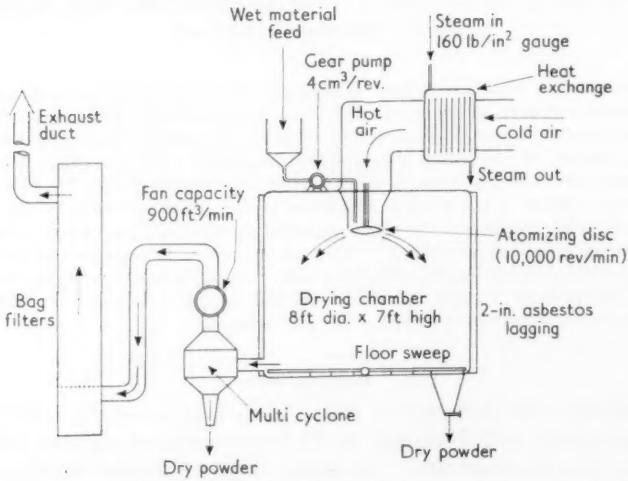

Fig. 1. Schematic layout of Scott spray-drying pilot plant (all product contact parts in stainless steel)

Spray-drying of Tomato Puree

Commercial spray-dried tomato powders have been available for some time but they have generally been of poor quality. We therefore investigated the spray-drying of tomato puree in order to establish the necessary conditions for the commercial production of more acceptable powders. We also investigated the cause of flavour deterioration in spray-dried tomato powders and this led to a study of the preparation of tomato purees with the object of improving the storage properties and flavour of the powder.

The quality of the powder was assessed from the amount of original pigment retained after drying and the amount of browning or caramelization which had taken place during drying (see

Appendix I for analytical method). Petroleum ether–water extraction was used to separate the original pigment (petrol-soluble colour) and pigments due to browning (water-soluble colour). The colours were computed as percentages of the ratio of the absorption coefficient and weight of the sample and were expressed as $E_{1cm}^{1\%}$ λ 472 mμ units for the petrol-soluble colours and $E_{1cm}^{1\%}$ λ 380 mμ units for the water-soluble colours.

Lazar[13] stated that it was very difficult, if not impossible, to spray-dry tomato juice without extensive modifications to a conventional spray-drier and then only under critically controlled humidity conditions. Our initial attempts to spray-dry good quality Greek tomato puree on the Scott pilot plant spray-drier were unsuccessful. The chamber wall was covered with partly dried puree with only a small quantity of powder on the floor. At the time it was thought that this was due to either (*a*) uneven loading of the disc resulting in poor atomization, or (*b*) thermoplasticity of the dried tomato puree. The uneven loading was reduced by fitting a perforated hollow ring to the outlet end of the feed pipe but this resulted in only marginal improvement. A water-cooled hollow plate attached to the chamber wall showed that chilling the material near the wall did not improve drying.

Satisfactory powders from commercial tomato puree were obtained by making a simple modification to the Scott pilot plant. The modification consists of forming the inlet ducting for the drying air into a cone within a cylinder. This increases the distance travelled by the atomized particles before they meet the incoming hot air. Fig. 2 shows the original and modified air inlet systems. In a 5-day run, with cleaning every 6 h, the total material loss was 15 per cent; 13½ per cent had stuck to various parts of the drier and had to be discarded. Table I shows the moisture content and colours of the Italian tomato puree and the spray-dried powder obtained from the 5-day run.

These results show that spray-drying causes negligible colour change.

The exhaust air temperature must not exceed 70°C when drying tomato puree; the rate of browning increases rapidly above 70°C. Fig. 3 shows the rate of browning of spray-dried tomato powder retained in the chamber at 60, 70 and 80°C; the powder can be

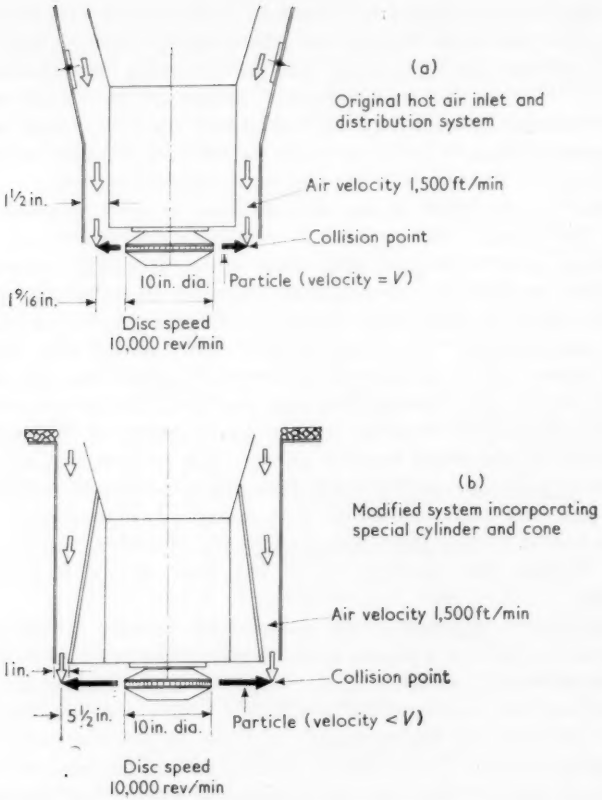

Fig. 2. Original and modified air inlet systems of Scott pilot plant

Table I. Moisture content and colour values of tomato puree and spray-dried powder. Colour value based on dry solids

Moisture content, %		Petrol ether-soluble ($E_{1cm}^{1\%}$ λ 472 mμ)	Water-soluble ($E_{1cm}^{1\%}$ λ 380 mμ)
Puree	71·1	4·65	0·345
Powder	2·6	4·42	0·394

kept at 70°C for up to 6 h without appreciably increasing browning but at 80°C the browning is high even after 30 min.

The colour values in Table I are for freshly dried powders but the values change with storage; the pigment value (petrol ether-soluble colour) decreases with time and the water-soluble or browning colour increases. There is an initial rapid change in the first day but the colour value decreases less rapidly over the next 6 months. There is some evidence that the keeping properties of the powder are related to cell damage in the puree. Microscopic examination of powders from commercial hot-break puree

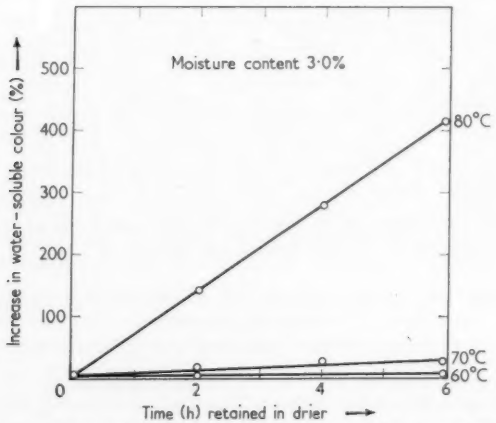

Fig. 3. Rate of browning of tomato puree at 60, 70 and 80°C

showed severe cell damage whereas powders from cold-break purees have only minor cell damage and in general have a longer shelf-life, better tomato flavour and colour.

Cells are damaged when the puree is made by the hot-break process; the tomatoes are steam-peeled, screened to remove skins and seed, heated to about 99°C and concentrated under reduced pressure. No heat is used in the cold-break process; the tomatoes are pulped, screened and then concentrated under vacuum at 30°C.

The powder particles from hot-break puree are irregular in shape and some have a glass-like outer shell enveloping an intense red-orange nucleus. There is some evidence that the greater the

number of glass-like particles the longer the powder will keep before bleaching.

Satisfactory powders can also be obtained by adding a carrier to the puree before spray-drying. Several carriers have been tried, including pectin, starch, sugar, gelatine, fat, sodium pectate, methyl ethyl cellulose (Edifas A), locust bean gum and commercial alginates. Gelatine proved to be the most suitable; good quality powders were produced with 15 per cent gelatine (on tomato solid) in puree diluted to 15 per cent solids.

Physical Characteristics of Spray-dried Tomato Powders

The physical characteristics of spray-dried powders obtained from solutions can be varied and controlled within certain limits by changing the drying conditions. Tomato purees, however, are not solutions and the characteristics of the puree also control, to some extent, the type of powder obtained. The powder from a free-flowing South African puree is finer than that from the thick Italian purees. Bulk density, determined by the constant volume method and by a free-fall method, and sieve analysis of spray-dried tomato powder from Italian puree are shown in Table II.

Table II. Bulk density and sieve analysis of spray-dried tomato powder from Italian puree

Bulk density		Sieve analysis, %			
Constant vol. method, lb/ft³	Free-fall method, lb/ft³	Larger than 60 mesh	60–80 mesh	80–200 mesh	Smaller than 200 mesh
46·5	35·4	8	13	49	30

Spray-dried tomato puree is a fine powder with a wide range of particle sizes. The bulk density is high, indicating that the particles are solid; this contrasts with the hollow particles of most spray-dried food products.

Spray-dried Groundnut and Soyabean Protein

Protein extracted from defatted groundnut and soyabean meal ferments in about 24 h at ambient temperatures and although it

keeps indefinitely at $-30°C$, storage at this low temperature is not economic. The spray-dried proteins, however, can be stored indefinitely at ambient temperature without deterioration.

Protein was extracted from groundnut or soyabean meal by aqueous sodium hydroxide at pH 7·5–10 and the extract was clarified by centrifuging. The protein was precipitated at its isoelectric point by adding hydrochloric acid to pH 5·5, and then concentrated by further centrifuging. The protein was spray-dried either as a slurry or solution above pH 6·8.

Proteins coagulate at high temperatures and initial attempts to dry groundnut and soyabean proteins were unsuccessful because the spinning disc atomizer quickly became clogged. This was overcome to some extent when the discs were coated with a silicone varnish (Midland Silicone Varnish MS1). The drying conditions are not so critical as for tomato puree, but when drying a slurry, the particle size must be below one thousandth of an inch; otherwise atomization and drying is inadequate. The right particle size can be obtained by colloid-milling the slurry at a gap setting of 0·005 in. One feature of groundnut protein is that it dries more efficiently at about three quarters the maximum available air throughput and the chamber exhaust temperature must be kept above 75° to avoid wet patches on the chamber wall. Even under the best conditions, the chamber wall was covered at the end of the run with dry and semi-dry protein but most of this could be brushed out. The experimental results quoted in this paper are for groundnut protein; similar results were obtained for soyabean protein. Typical drying conditions are shown in Table III.

The distribution of powder in the various sections will vary from drier to drier and in general can be established only by carrying

Table III. Conditions for spray-drying groundnut protein slurry

Slurry concentration (% solids)	24·5
Throughput (lb slurry/h)	53
Evaporation rate (lb water/h)	40
Temperatures (°C)	
Air inlet	152
Exhaust air from chamber	75
Chamber wall	69
Air throughput (ft^3/min)	600–650

out a test run. Table IV shows the proportions of protein powder collected continuously by mechanically sweeping the chamber floor, retained in the multicone cyclone, and swept out manually from the chamber (including wall deposits), when 25 per cent groundnut protein slurry at the isoelectric pH 5·4 and groundnut protein solution at pH 7·5 were spray-dried in the Scott pilot plant drier.

Table IV. Spray-drying of groundnut protein slurry and solution: quantities of powder collected in sections of Scott pilot plant spray-drier

	Proportions collected, % total		
	Continuous	Cyclone	Swept out
Slurry	43·4	22·6	34·0
Solution	41·2	29·4	29·4

The quantities collected in each section are about the same for slurry and solution, but the particle size distributions (determined by sieving through analytical sieves for 1 h) are significantly different, as shown in Table V; especially the powders collected continuously and swept out at the end of the run.

Table V. Sieve analysis of spray-dried groundnut protein

			Percentage retention	
			Slurry	Solution
Collected continuously	Retained by	60 mesh	21·5	2·0
		100 mesh	7·5	20·6
		200 mesh	23·5	21·4
	Through	200 mesh	47·5	56·0
Collected in cyclone	Retained by	60 mesh	1·3	1·0
		100 mesh	2·6	—
		200 mesh	26·5	41·0
	Through	200 mesh	69·6	58·0
Swept out manually	Retained by	60 mesh	33·5	10·0
		100 mesh	12·5	20·1
		200 mesh	20·5	30·6
	Through	200 mesh	33·5	39·3

The relatively high percentage retained on 60 mesh from the continuous and swept fractions is due partly to the particle size of the slurry and partly to agglomerates formed during drying.

The bulk density of groundnut protein powder from slurry is 50·4 lb/ft^3 (constant volume method) and 39·9 lb/ft^3 (free-fall method); the value for powder from solution is 45 lb/ft^3 (constant volume method).

Effect of Spray-drying on the Viscosity and Colour of Groundnut Protein

Little is known about the effect of spray-drying on groundnut protein. The viscosity and colour of solutions of the dried protein at above pH 10 and its nutritive value were therefore investigated in some detail.

Viscosity and Colour of Groundnut Protein Solutions

Groundnut protein in alkaline solution above pH 10 is brownish-red and viscous; the viscosity increases with time.[14] Such solutions form filaments when extruded through spinnerets into an acid coagulating bath. The normal method for producing such filaments is to dissolve the protein in an aqueous alkaline medium at pH 12 and then age the solution for 24 h or more. During this time, the viscosity increases gradually and continuously to a peak (known as peak viscosity) at which point the solution is extruded. This method was modified so that the peak viscosity is reached in a matter of minutes.[15] Table VI shows the peak viscosity and time to reach peak viscosity of a spinning solution prepared from the same batch of protein before and after spray-drying. The viscosity was measured by a viscometer using a Teflon bob rotating at 5 rev/sec.

Table VI. Viscosity of groundnut protein solution before and after spray-drying

	Peak viscosity, poise at 5 sec^{-1}	Time required to reach peak viscosity, min
Before spray-drying	40	8·0
After spray-drying	60	8·0

Spray-drying, using air drying temperatures between 75–100°C, had little effect on the viscosity or the time to reach peak viscosity.

Table VII shows colour values of a spinning solution prepared from the same batch of extracted protein before and after spray-drying.

Table VII. Colour values of a groundnut protein spinning solution before and after spray-drying (colour measured on Lovibond–Schofield Tintometer)

	Colour value		
	Red	Yellow	Brightness
Before spray-drying	3·8	14·0	1·1
After spray-drying	3·9	14·1	0·9

Spray-drying did not significantly affect the colour of the solution.

Nutritive Value of Groundnut Protein

Nutritive value was assessed by rat growth. Groundnut protein was fed as sole source of protein at 14 per cent in purified diets to young Colworth rats of Wistar origin; experience has shown that 14 per cent protein produces suboptimal growth and is satisfactory for the detection of small differences in nutritive value.

Table VIII shows the results of a feeding trial in which 3 groups of 12 rats derived from 12 litter-mate trios and composed of equal numbers of bucks and does were fed diets containing spray-dried, alcohol-dried or freeze-dried groundnut protein for 3 weeks. The diet used is shown in Appendix II.

Table VIII. Nutritive value of dried groundnut protein

Method of drying the protein	Average initial wt., g	Average gain 3 weeks, g	Average accumulated food intake, g	Average accumulated protein intake, g	Average gain/unit food intake, g	Protein efficiency[a]
Spray	54·2	53·0	224·8	31·5	0·235	0·59
Alcohol	54·8	51·7	226·2	31·6	0·227	0·61
Freeze	53·5	47·0	209·4	29·3	0·224	0·60

[a] Protein efficiency = average weight gain/average protein intake.

Spray-drying has no adverse effect on the nutritive value.

These results show that spray-drying, even at the relatively high chamber temperature of 80°C, has little effect on groundnut protein. Similar results have been obtained with soyabean protein.

Sugar–Gelatine Solution

Gelatine solutions are difficult to spray-dry because they tend to form filaments rather than droplets when atomized. Gelatine dried in this manner is soluble in water at 40°C but not in cold water. When sugar–gelatine solutions are spray-dried they produce a powder that is fairly easily dispersed and dissolved in cold water. The sugar–gelatine powder–water mixture forms a light foam-like jelly when it is whisked during reconstitution. However, the

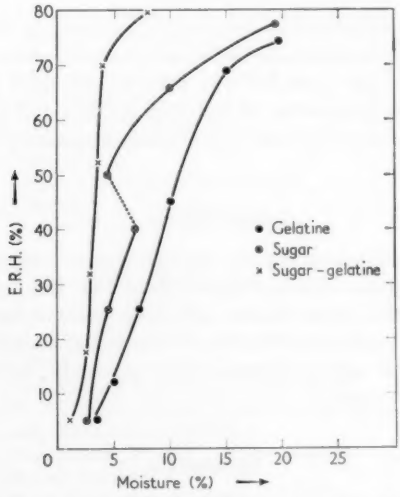

Fig. 4. ERH moisture relation of spray-dried sugar–gelatine mixture

degree of foaming varied considerably from batch to batch even when processing and drying conditions were nominally the same; this lack of reproducibility could not be explained.

The equilibrium relative humidity–moisture curves (ERH curves) for gelatine and sugar are smooth and continuous but there is a marked and sudden change in the curve of spray-dried sugar–gelatine powder at about 7 per cent moisture (see Fig. 4). This may be an hysteresis effect but this phenomenon has not yet been studied in detail.

The spray-drying conditions were somewhat critical; typical conditions are shown in Table IX.

Table IX. Spray-drying conditions for sugar–gelatine solutions

Drying air rate: 1,400 ft³/lb feed
Chamber air inlet temperature: 140°–156°C
Chamber air exhaust temperature: 68°–75°C
Feed solids content: 20%

Exhaust air temperatures below 67°C resulted in sticky deposits on the chamber wall and the powder browned above 75°C. The bulk density of the powder was low: about 26·0 lb/ft³ by the constant volume method or 18 lb/ft³ by the free-fall method. The powder was free-flowing and only slightly hygroscopic.

Conclusions

This review of spray-drying tomato puree, vegetable proteins and sugar–gelatine solution shows that the temperature in the spray-drier is the major factor affecting the quality of the final powder. One cannot predict from theoretical considerations how spray-drying will affect different food products; a trial run must be made on the plant.

Appendix I

Determination of Tomato Powder Colour

The determination is carried out in diffused light. About 0·1 g of powder is accurately weighed into a stoppered 100-ml graduated cylinder. About 20 small glass beads and 35 ml of hot distilled water are added and the cylinder is shaken to dissolve the powder; 15 ml of 95 per cent ethanol and 50 ml petrol ether (b.p. 80–100°C) are added and the cylinder is shaken mechanically for 1 h. After standing to separate the petrol ether and water phases, a sample pipetted from the petrol ether layer is filtered through a plug of absorbent cotton wool and a sample pipetted from the lower aqueous layer is filtered through a double fluted filter (Whatman No. 42 and No. 542). The absorption coefficients of the filtrates are determined at λ 472 mμ for the petrol ether sample against a blank of the same petrol ether, and at λ 380 mμ for the aqueous sample against a blank of

distilled water, both in 1-cm cells. The extinction coefficients are calculated as:

$$E_{1\text{cm}}{}^{1\%} = \frac{e \times 100}{TS \times W \times 2}$$

where e = absorption coefficient of the sample, W = weight of sample taken, and TS = % tomato solids content of sample, allowance being made for moisture content and any additives. Moisture content is determined by means of the Karl Fischer titration method. A Unicam SP 600 spectrophotometer was used for measuring optical densities.

Appendix II

Composition of groundnut diet	%
Vitamin B mixture	5
Cellulose powder	4
Hubbell's salt mixture	4
Groundnut oil	10
Wheat germ oil	0·5
Protein from groundnut sample	14
Starch	to 100

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Ion-Exchange Chromatography of the Streptomycin Complex

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Summary. Various studies have revealed the presence of several maltol-producing compounds in streptomycin preparations. These previous studies have utilized the techniques of counter-current distribution, column chromatography, paper chromatography, and electrophoresis. A system of cation-exchange chromatography has been reported which separates, at least partially, six components of the streptomycin complex. This technique involves the use of a gradient elution procedure.

In addition to five maltol-producing compounds, another member of the complex is reported. The possible significance of such a compound is discussed.

Introduction

The studies of Fried and Titus,[1] and Titus and Fried[2] have revealed the presence of several maltol-producing compounds in streptomycin preparations. By the use of the Craig counter-current distribution technique and chromatography on acid-washed alumina, mannosidostreptomycin was detected. Further work by Titus and Fried[3] revealed another compound which they considered a tautomer of streptomycin. The presence of a third component was verified by the paper partition chromatography studies of Winsten and Eigen.[4]

More recently, the use of paper electrophoresis by Foster and Ashton[5] has provided another technique for the separation of some of the components of the streptomycin complex.

Fardig and Hooper[6] have achieved a partial separation of streptomycin and mannosidostreptomycin by a preliminary elution from a cation-exchange resin with a boric acid solution. This technique removed approximately 50 per cent of the mannosidostreptomycin present in crude mixtures.

This report describes a system of ion-exchange chromatography which separates streptomycin, mannosidostreptomycin, and

several other maltol-producing components of the streptomycin complex.

Experimental

Culture

The streptomycin mixtures used in this work were recovered from a culture of *Streptomyces griseus* known to produce a high proportion of mannosidostreptomycin. The recovery technique described by Friedman and his associates[7] was used to prepare two streptomycin mixtures.

Mixture 1. A streptomycin mixture high in mannosidostreptomycin content was obtained by isolating streptomycin from a calcium chloride double-salt mother liquor. This mixture had a biological activity of 285 μg/mg and contained 37 per cent mannosidostreptomycin.

Mixture 2. This mixture of streptomycins was prepared by the ion-exchange procedure followed by methanol precipitation. The mixture had a biological activity of 665 μg/mg and contained 8 per cent mannosidostreptomycin.

Preparation of Resin

Amberlite CG–50 Type II (200–400 mesh) with 4–6 per cent divinylbenzene cross-linkage was used throughout. The resin in the hydrogen form was suspended in three volumes of water for 2 min and decanted in order to remove large particles. The resin was then suspended in three volumes of water and allowed to settle for 1 h after which the supernatant was decanted. This step was repeated until all of the fine particles had been removed. The resin was suspended in three volumes of 1 M NaOH for conversion to the sodium form. The sodium resinate was then washed with water until the reaction of the wash water was neutral.

The lack of precision of the maltol assay below a streptomycin concentration of 200 μg/ml required that relatively large amounts of streptomycin complex be used. This requirement made necessary the use of large columns to avoid overloading the resin. Columns of 2·54 cm by 76·0 cm dimensions were poured and used throughout.

Operation of the Columns

Streptomycin (1·5 g) was dissolved in water (5 ml) and absorbed on the resin as a narrow band at the top of the column. In the early part of the work a 0·85 M NaCl elution solution was used. Although separations were possible with this agent, excessive tailing of the peaks tended to obscure large portions of the chromatograms. A gradient elution system consisting of a reservoir of 1·20 M NaCl which emptied into a 2-l. reservoir of 0·50 M NaCl was used. By this procedure, the molarity of the eluting agent was constantly increased and the tailing difficulty was reduced.

The effluent was collected in 20-ml fractions at a flow rate of 1·4 ml/min.

Assays

All fractions were subjected to analysis by the maltol assay[8] and anthrone assay.[9] Selected fractions were analysed by paper chromatography essentially as described by Winsten and Eigen,[4] except that a 2 per cent solution of *p*-toluenesulphonic acid in moist butanol was used and the indicator organism was *Bacillus subtilis*. Biological activity of selected fractions was determined by the cylinder-plate method[10] using *B. subtilis* as the test organism.

Results and Discussion

As can be seen in Fig. 1, a plot of maltol assay against fraction numbers revealed the presence of five maltol-positive peaks in mixture 1. At least a partial separation of the five maltol-producing compounds was achieved. Paper chromatography of the peak fractions indicated that all five compounds had some degree of biological activity against the indicator organism. Table I presents the chromatographic characteristics of these compounds on cellulose sheets.

Biological assays of all fractions disclosed the various biological activities of the maltol-producing compounds. The R_s value, close agreement between biological activity and maltol-producing ability, and lack of a positive anthrone reaction indicate clearly that peak IV represents streptomycin itself. From the relative heights of the peaks of maltol activity and biological activity, it

can be seen that all of the compounds are less active than the streptomycin of peak IV. The ratios of biological activity to maltol activity are presented in Table I.

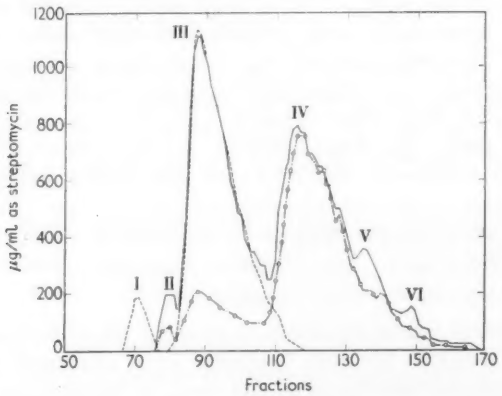

Fig. 1. Chromatogram of streptomycin preparation containing 37 per cent mannosidostreptomycin (mixture 1). Maltol assay—solid line; anthrone assay—broken line; biological assay—linked circles

Table I. Summary of peak characteristics. 37 per cent mannosidostreptomycin preparation

Fraction no.	R_f value,[a]	Biological assay	
		Maltol assay	Anthrone assay[b]
71	—[c]	—[d]	190
80	0·18	0·400	0
88	0·38	0·187	1150
118	1·00	0·962	10
136	0·58	0·667	0
150	0·32	0·533	0
Mannosidostreptomycin standard	0·37	—	—
Hydroxystreptomycin standard	0·64	—	—
Dihydrostreptomycin standard	0·62	—	—

[a] Ratio of distance travelled by compound/distance travelled by streptomycin.
[b] Calculated as mannosidostreptomycin.
[c] Lacks biological activity.
[d] Lacks biological activity and maltol activity.

Anthrone assays of all fractions detected the presence of two anthrone-positive compounds. Based on the positive anthrone test, the bioassay to maltol assay ratio, and paper chromatography, the second anthrone peak (peak III) of Fig. 1 must represent mannosidostreptomycin.

The smaller anthrone-positive peak (peak I) lacks biological activity and maltol-producing ability. Such a compound has not been previously reported and may represent a carbohydrate degradation product such as free mannose, although such a degradation product is not observed in acidic eluates from cation-exchange resins. The use of a neutral eluting agent tends to eliminate this possibility. Compounds such as streptidine, *N*-methyl-L-glucosamine, and streptobiosamine do not give positive anthrone reactions and can be excluded from consideration. Another possibility would be a compound containing a cationic group which adsorbs on the resin and a carbohydrate moiety, the presence of which causes a positive anthrone reaction. A compound similar to this description, thymidine diphosphate mannose, recently isolated by Baddiley and Blumson,[11] occurs in the mycelium of *Streptomyces griseus*. However, since thymidine diphosphate mannose would adsorb weakly to a cation-exchange resin, it is probably not identical with the unknown anthrone-reacting compound detected in this study. A compound similar to either D-mannosyl-α-*N*-methyl-L-glucosaminide or a mannosidostreptomycin in which the streptose moiety is blocked may be involved. The isolation and determination of the structure of this compound would provide a basis for a choice between these possibilities. Work of this nature is continuing in this laboratory.

Peak II (fraction 71) has maltol-producing ability and biological activity but lacks a positive anthrone assay. The R_s value of this compound (0·18) is quite different from that of other known streptomycin-like entities such as hydroxystreptomycin and dihydrostreptomycin. The component of this peak was also found in the originally pure streptomycin of peak IV (fraction 118) after it had been stored at 4°C for ten days. This behaviour of the compound indicates that it may be identical with one of the tautomeric forms of streptomycin described by Titus and Fried.[3]

The compound detected in peak V migrates in the region of

dihydrostreptomycin and hydroxystreptomycin on paper chromatograms. Fortifying fraction 136 with a dihydrostreptomycin standard increased the inhibitory area of the unknown spot. However, since dihydrostreptomycin does not give a positive maltol test, it cannot be identical with the material of fraction 136. A compound which migrated between streptomycin and mannosidostreptomycin was detected by Winsten and Eigen[4] and appears to be similar to the one reported in this study.

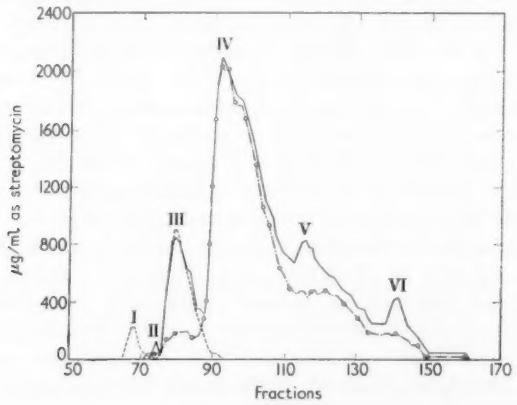

Fig. 2. Chromatogram of streptomycin preparation containing 8 per cent mannosidostreptomycin (mixture 2). Maltol assay—solid line; anthrone assay—broken line; biological assay—linked circles

The unknown compound of peak VI (fraction 150) migrated on a paper chromatogram in the region just below mannosidostreptomycin. The behaviour of this compound on chromatograms did not correspond to that of known streptomycin-like standards. The compound was not observed in streptomycin fractions which had been stored and probably does not represent a tautomeric form. Winsten and Eigen[4] reported the presence of a similar compound in a residue from the purification of streptomycin. This compound behaved exactly as the unknown of fraction 150 on paper chromatograms. No details as to the purification technique were provided by these authors, although it is likely that the calcium chloride double-salt method was used.

If this is the case, the material of peak VI is probably the same as that of Winsten and Eigen.

The results obtained with the use of a much more highly purified streptomycin preparation (mixture 2) were similar to those obtained for the high mannosidostreptomycin mixture (mixture 1) except that the relative proportions of some of the peaks were different. Six peaks were detected in this mixture by the use of the anthrone, maltol, and biological assays (Fig. 2). As would be expected, the amount of mannosidostreptomycin in relation to that of streptomycin is much less than in the mixture derived from calcium chloride double-salt mother liquors. The relative area under peak II is much less in the case of this mixture, indicating that this compound does not form a double salt and is therefore recoverable from double-salt mother liquors.

Paper chromatography of the components of mixture 2 was very similar to that of mixture 1 and therefore the data presented in Table I are also representative of the results obtained with mixture 2.

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Recovery of Bacterial Spores and Vegetative Cells with a Miniature Spray Drier

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Summary. A laboratory spray drier with a gas flow of 2 to 3 ft^3/min was used to dry suspensions containing spores of *Bacillus anthracis* or cells of *Pasteurella tularensis*. Dried material was collected in a ½-in. diameter cyclone that retained about 97 per cent of the product from the drier. The particle size distributions of the powders varied with the nature of substances in the suspension to be dried; as much as 80 per cent of the mass in particles 5 μ or less in diameter was obtained only when egg yolk was added to the suspensions. Retention of viability was about 90 per cent for *B. anthracis* spores and these powders contained from 175 to 522×10^9 viable spores/gram. Retention of viability of *P. tularensis* cells was 0·6 per cent or greater only when the suspension contained a relatively high proportion of stabilizers. With sucrose–skim milk–thiourea stabilizer about 10 per cent recovery of viability was obtained when the proportion of stabilizer solids to other solids was 3 : 1 or 6 : 1. These powders contained 140×10^9 viable cells per gram and the mass median diameter was 6·0 μ. The properties of the powders suggest that spray dried vaccines may be prepared for use either in suspension or in aerosols.

Spray-drying has potential advantages in the preparation of biological products used as vaccines or insecticides because it would probably be economical and the spray-dried powder might be suitable for use with no further processing. This would be true particularly for biological products used as dusts and aerosols, such as dried *Bacillus thuringiensis* Berliner used as an insecticide,[1] and various aerogenic vaccines. Vaccine against Newcastle Disease has been administered to poultry in clouds of dried material for approximately a decade,[2] and further interest in aerogenic vaccines is evidenced by recent reports. For example, Aleksandrov *et al.*[3] found that dry, living aerogenic vaccine was effective against anthrax and offered advantages for mass vaccination of people and farm animals. Saslaw *et al.*[4, 5] and Eigelsbach *et al.*[6, 7] reported that in both laboratory and clinical trials

dried living anti-tularemia vaccine was more effective than non-living vaccine. In addition, from recent studies with monkeys and guinea pigs, Eigelsbach *et al.*[8] observed that with living vaccine, aerogenic vaccination afforded greater immunity against tularemia than did vaccination by the dermal route.

The possibility that dried live vaccines might be prepared with a spray drier arises from studies conducted at the University of Wisconsin with 'model' vegetative organisms and from more recent work with tularemia- and anthrax-causing organisms. Splittstoesser and Foster[9] reported high recovery of viable *Serratia marcescens*, *Bacterium linens*, *Escherichia coli*, *Micrococcus ureae*, and *Streptococcus lactis* in simulated spray-drying conditions. The Wisconsin group also obtained high recovery (about 60 per cent) of viable *S. marcescens* in a spray drier.[10] This paper reports studies on spray drying of *Pasteurella tularensis* and *Bacillus anthracis* and it may be expected that vaccine strains of these organisms could be dried with results similar to those reported here.

The miniature spray drier used in this work was developed by Garver and Tschernitz at the University of Wisconsin.[10] As shown in Fig. 1, the drier consists of gas heating, spray mixing, drying, and collecting sections. The gas heating section is 3 in. in diameter by 18 in. long and heat is supplied by four strip-type electric heaters. The spray mixing section is 4 in. in diameter by 6 in. long, and a funnel-shaped unit is used to facilitate envelopment of the spray by the hot gas. The drying section is 4 in. in diameter by 24 in. long. The collecting section is tapered to a $\frac{1}{4}$-in. connection to a cyclone.

The suspension to be dried is atomized in a Chicago Toxicity Laboratory-type atomizer and the spray is introduced into the hot gas through a right-angled tube and the funnel unit. In our work, dried material was collected with $\frac{1}{2}$-in. diameter cyclones of the standard design adopted by General Mills personnel for collecting ground materials.[11] The drier and cyclone system was operated with a vacuum pump connected to the exhaust of the cyclone and the flow was regulated so that the pressure at the inlet to the cyclone was about $-\frac{1}{2}$-in. Hg. In tests in which two cyclones, a paper filter, and a glass fibre filter were used in series, 97 per cent of the total material collected was retained in the first

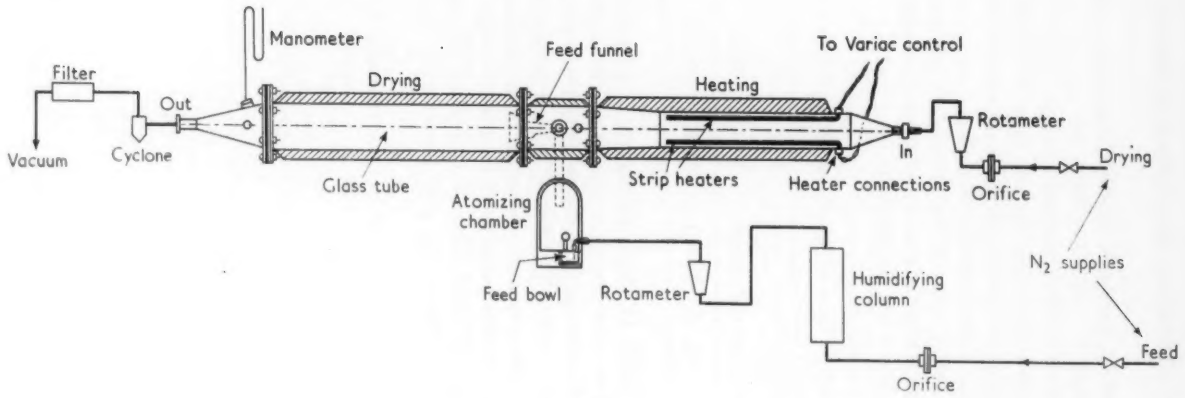
Manometer
Filter
Out
Drying
Feed funnel
Heating
To Variac control
Rotameter
Vacuum
Cyclone
Glass tube
Strip heaters
Heater connections
In
Orifice
Drying
Atomizing chamber
N_2 supplies
Feed bowl
Rotameter
Humidifying column
Feed
Orifice

Fig. 1

cyclone. Therefore, only one cyclone was used routinely to collect dried material.

Particle-size distributions of the dried materials were measured by the Whitby centrifuge method.[12] The fluid systems for dispersion and settling of the powders were kerosene and petroleum ether with 0·2 per cent naphthenic acid.

The gas-flow rates, rates of feed, and inlet and outlet temperatures were selected from the work at Wisconsin and were varied only slightly. These are shown in Table I.

Table I. General operating conditions for laboratory spray drier

Nitrogen flow, ft^3/min		Temperature, °C		Feed rate, ml/min
Through atomizer	Through heater	Inlet	Outlet	
0·5–0·7	2·0–3·0	68–72	46–50	0·2–0·3

As shown in Table II, the concentration of viable spores in the dried powders from water suspensions ranged from 281 to 633 × 10^9 per gram, and percentage recoveries were from 40 to 148. The highest recoveries probably indicate that dispersion of the spores in suspensions of the powders was better than the dispersion in the control samples of the suspensions to be dried.

The data given in Table III indicate that the use of several materials called dispersers did not appreciably change the out-

Table II. Properties of *Bacillus anthracis* spray-dried from water suspensions

Viable spore concentration, 10^9/g	Viable spore recovery, %	Mass median diameter, μ	Total solids in feed suspension, %
613	63	7·1	1·33
566	40	6·1	1·23
585	97	5·2	3·40
281	79	10·1	2·74
423	63	9·4	3·65
502	140	6·1	3·12
479	92	12·9	4·35
613	148	12·3	3·30
633	124	6·0	1·42
Average 522	94	7·9	2·73

come of the drying process. Table IV shows an interesting effect on the particle size of the dried powders. In spite of the high solids content of the feed to the driers, these powders, prepared from suspensions containing egg yolk, all had mass median diameters below 5 microns. A summary of the properties of spray-dried *B. anthracis* materials is shown in Table V.

Table III. Properties of *Bacillus anthracis* dried from suspensions with 'dispersers'

'Disperser'	Viable spore concentration, 10^9/g	Viable spore recovery, %	Mass median diameter, μ	Total solids in feed suspension, %
Tamol N	414	70	12·1	4·39
Tamol N	546	93	8·7	3·21
Diglycol laurate	542	84	10·1	4·26
Diglycol laurate	620	94	6·6	3·44
Aerosol OT	521	104	12·8	3·18
Lecithin (animal)	438	75	12·9	3·10
Average	514	87	10·6	3·60

Table IV. Properties of *Bacillus anthracis* spray-dried from suspensions containing egg yolk

	Viable spore concentration, 10^9/g	Viable spore recovery, %	Mass median diameter, μ	Total solids in feed suspension, %
	188	104	3·6	5·46
	3	105	4·4	6·39
	246	75	3·6	4·44
	163	96	3·9	5·52[a]
	277	126	2·7	4·67[a]
Average	175	101	3·6	5·30

[a] Suspensions also contained GE–81648 antifoam.

Table V. Summary of properties of spray-dried *Bacillus anthracis* preparations

Feed suspension	Viable spore concentration, 10^9/g	Viable spore recovery, %	Mass median diameter, μ	Total solids in feed suspension, %
Water	522	94	7·9	2·73
Water + 'dispersers'	514	87	10·6	3·60
Water + egg yolk	175	101	3·6	5·30

The conditions for spray drying *P. tularensis* were similar to those for *B. anthracis*, except that lower temperatures were used initially. Inlet and outlet temperatures of 60° and 40° were used in the first trials because it was assumed that the sensitive vegetative cells would withstand the lower temperatures better than the higher ones. These temperatures were gradually increased to 70° and 48°C. Gas-flow rates were 0·5 ft^3/min through the atomizer and 2·0 ft^3/min through the heater. The work with *P. tularensis* was less extensive than that with *B. anthracis*, but, as shown in Table VI, recovery of viable cells was increased more than a hundred-fold by proper use of stabilizers.

Table VI. Properties of spray-dried preparations of *Pasteurella tularensis*

Stabilizer	Ratio of stabilizer to other solids	Viable cell concentration, 10^9/g	Viable cell recovery, %	Mass median diameter, μ	Total solids in feed suspension, %
None	0:2	0·5	0·02	—	1·98
Raffinose	0·6:1	0·5	0·02	—	2·74
Glucose	0·6:1	0·4	0·02	—	2·74
Glucose	4:1	6·0	0·6	6·3	4·71
SST[a]	3:1	146	6·0	5·6	4·04
SST	6:1	135	14·0	6·5	6·08

[a] A mixture of sucrose, skim milk, and thiourea in the proportions 6:2·5:1.

Both the relative concentration and the nature of the stabilizer affected the percentage recovery of viable cells. When the relative concentration of glucose to other solids was increased from 0·6 : 1 to 4 : 1, recovery increased more than thirty-fold, to 0·6 per cent. The sucrose–skim milk–thiourea mixture seems to be the best stabilizer used since a 3 : 1 proportion of stabilizer gave a 6 per cent viable cell recovery, compared with 0·6 per cent for glucose in a 4 : 1 ratio. Twice as great a recovery was obtained with the 6 : 1 mixture.

These observations with a sensitive vegetative cell are too limited to permit firm conclusions to be drawn. However, in our opinion, they suggest that results of laboratory studies on spray drying of micro-organisms will be similar to the results of studies on freeze drying; i.e. with further use of easily manipulated

laboratory driers, with control of all phases of the procedure, and with proper use of stabilizers, recovery of viable organisms in spray-dried preparations will rapidly increase to nearly 100 per cent.

In summary, a laboratory spray drier developed by University of Wisconsin personnel and small cyclones of standard design have been useful for investigations on recovery of *B. anthracis* spores and *P. tularensis* cells.

Retention of viability of spores of *B. anthracis* was about 90 per cent. The particle-size distributions of the powders varied with the nature of substances in the suspensions to be dried. Powders with as much as 80 per cent of the mass in particles 5 microns or less in diameter were obtained only when egg yolk was added to the feed suspensions. These powders contained from 175 to 522×10^9 viable spores per gram. Recovery of viable cells of *P. tularensis* was 0·6 per cent or more only when a relatively high proportion of stabilizer was added to the cell suspension for drying. With a sucrose–skim milk–thiourea stabilizer, about 10 per cent recovery was obtained when the proportion of stabilizer solids to other solids was 3 : 1 or 6 : 1. These powders contained 140×10^9 viable cells per gram and the mass median diameter was 6·0 microns.

The properties of the dried powders suggest that spray-dried vaccines may be prepared suitable for use either in suspension or in aerosols.

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Mathematical Models for the Product Accumulation in Microbiological Processes

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Summary. A production model consisting of one or more exponential decay functions is proposed to represent the rate of accumulation of a product per unit weight of cell. This, together with a cell age distribution function, is used to describe the product accumulation in a fermentation process.

This postulate offers a unified concept applicable to both batch and continuous processes as well as problems arising from environmental changes. The idea is compatible with the current understanding of microbial physiology. It offers an interpretation for the controversy over cell-product ratios observed in some batch and continuous fermentations when treated as conventional chemical reaction. The proposed postulate fits reasonably well with the lysine and lactic acid processes.

Introduction

A fermentation process is conventionally described by the time response of the formations of cells and other products. The growth of the organism is divided into a number of phases. The progress of the process (i.e. the magnitude of product accumulation, exhaustion of substrates and the variations in hydrogen concentration and other environmental factors) is often discussed with reference to the growth phase of the culture. Such elimination of clock time is considered to be more effective in bringing out the characteristics of the organism and the process. Chemical and morphological changes which occur during the process are qualitatively interrelated or interpreted through the 'physiological state' of the culture as a whole. The effect of chemical and physical environments on the state of the cells has been of major interest for the workers in this field. Often the 'physiological state' is considered as a reflection of environmental conditions. The physiological age of an individual cell itself, however, has not received adequate attention in industrial practice.

There is a great deal of evidence indicating the existence of natural aging phenomena for cells of micro-organisms even though they are primitive. The growth activity of fungi mostly occurs at the tip of a mycelium. A yeast cell retains a bud scar after giving birth to each daughter cell; buds are not formed at the scarred area and the number of births per cell is thus limited.[1] Volume growth takes place mostly in the bud portion of the mother cell or the newly formed daughter cells.[2] Such localization of growth in mycelium, the labelling of cells by birth marks, and the time variations of biochemical activity,[3] definitely establish the identity, and consequently the history, of a particular cell. Bacterial cells which multiply by fission have been reported to exhibit time variations in cell volume,[4] temperature response[5] and multiplication rate.[6] Changes with time in physical and chemical properties of cells such as electro-kinetic mobility, metabolic rate, dye reducing capacity, ribonucleic acid content and the susceptibility to injurious chemical agents,[4, 7–9] have also been reported. These observations lead to the belief that each cell in a culture retains its identity and carries out its metabolic function according to a predetermined genetic order analogous to differentiated cells of higher life. In other words, under a given constant environment, each individual cell makes a product at a rate depending on the age of the cell material. Thus, there must be an age–production rate relationship to represent the production performance of individual cells in a given population. This basic pattern reflects the make-up in the metabolic pathways of the organism, which is in turn susceptible to a varied degree of modification by environmental changes. The variation in environmental factors such as temperature, pH and nutrient concentration may force the cells to shift the basic pattern to a new level or, in extreme cases, to adopt a different pathway.

From the foregoing discussion, it is reasonable to suggest that the physiological state of a culture as a whole is simply a statistical representation of the physiological state of individual cells of the entire population. It is apparent, then, that if the basic pattern of product formation by an individual cell can be represented in a quantitative form, the overall product accumulation may be estimated from the cell-age distribution of the population in the vessel. Thus, by the use of such a transformation of co-ordinates,

the direct dependence of product yield on the process time is eliminated.

The performance of a process expressed in this form should be applicable equally well to both batch and continuous processes. A transformation of this nature is useful in predicting the performance of a continuous process from batch data and also offers a new analytical approach to the study of microbiological processes.

While the present state of experimental technique is not adequate to explore the behaviour of individual cells in a mass culture, a generalized model of the product formation in a microbial cell is proposed and discussed in this paper.

Proposed Model for Product Accumulation

Consider a cell as a micro-reactor in which the product of interest is synthesized and destroyed. The resultant instantaneous rates of these activities under a constant environmental condition are assumed to take the form:

$$r_p = \frac{dp}{d\theta} = \sum_i A_i e^{-k_i \theta} \tag{1}$$

where A_i is a rational number and k_i a positive number; these are defined by the characteristics of the organism and the environment.

It follows, then, that the amount of product made by unit weight of a cell in a period θ of its life span is:

$$p_0 = \int_0^\theta \sum_i A_i e^{-k_i \theta} d\theta \tag{2}$$

If the cell-age distribution in a process vessel covers a range 0 to t, and the concentration of cells age θ is represented by a function $C(\theta)$, such that the overall cell concentration in the vessel is

$$C_t = \int_0^t C(\theta) d\theta \tag{3}$$

then the overall product concentration in the vessel containing cells with an age spread between 0 and t may be described as

$$P = \int_0^t C(\theta) \int_0^\theta \sum_i A_i e^{-k_i \theta} d\theta d\theta \tag{4}$$

In the case where the product does not appear until after the age reaches a value $\bar{a}$, equation (2) will become

$$p_\theta = \int_{\bar{a}}^{\theta} \sum_i A_i e^{-k_i\theta} d\theta \tag{5}$$

and equation (4) becomes

$$P = \int_{\bar{a}}^{t} C(\theta) \int_{a}^{\theta} \sum_i A_i e^{-k_i\theta} d\theta d\theta \tag{6}$$

Various modes of product formation by a cell may be described by an appropriate choice of the i, k_i and A_i values for equation (1).

The single exponential decay equation

$$r_p = Ae^{-k\theta} \tag{7}$$

may be used to describe a rate process completed in a relatively short or long period of its life span by employing large or small k values respectively. A negative term with a relatively large coefficient and decay constant is introduced to represent a process of delayed production, i.e.

$$r_p = A_1 e^{-k_1\theta} - A_2 e^{-k_2\theta} \tag{8}$$

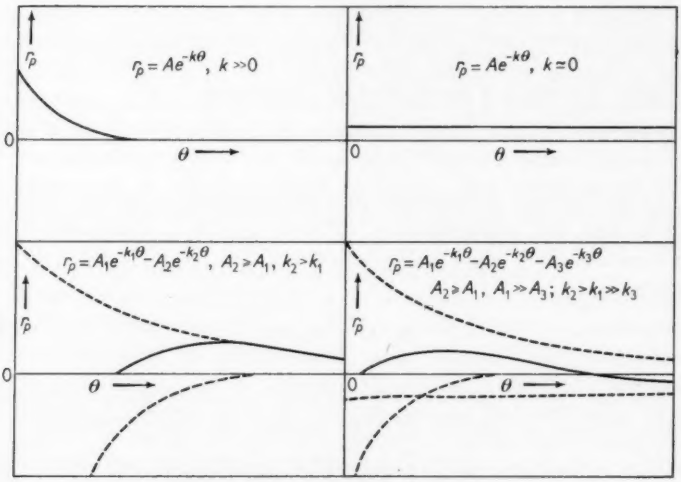
$r_p = Ae^{-k\theta}$, $k \gg 0$
$r_p = Ae^{-k\theta}$, $k \simeq 0$
r_p
0
θ
$r_p = A_1e^{-k_1\theta} - A_2e^{-k_2\theta}$, $A_2 \geq A_1$, $k_2 > k_1$
$r_p = A_1e^{-k_1\theta} - A_2e^{-k_2\theta} - A_3e^{-k_3\theta}$
$A_2 \geq A_1$, $A_1 \gg A_3$; $k_2 > k_1 \gg k_3$

Fig. 1. Examples of models for product accumulation

where

$$A_2 \geq A_1; \quad k_2 > k_1.$$

An additional negative term with a relatively small coefficient and a small decay constant, as shown in equation (9), is suitable for describing transient accumulation with a delayed start:

$$r_p = A_1 e^{-k_1\theta} - A_2 e^{-k_2\theta} - A_3 e^{-k_3\theta} \tag{9}$$

where

$$A_2 > A_1 \gg A_3 \quad \text{and} \quad k_2 > k_1 \gg k_3.$$

Graphical representations of equations (7), (8) and (9) are shown in Fig. 1.

Approximations of the most commonly encountered process patterns may be covered by equations (7) to (9).

Age Distribution in Batch Processes

The age distribution in a population of cells in a batch process may be derived from the cell concentration–time relation. The growth rate in various phases may be represented by appropriate equations. It is then possible to find the concentration function at any age θ counting from the beginning of the particular phase under consideration. For instance, if the instantaneous growth rate is represented by $R(t)$, then it is obvious from Fig. 2 that the

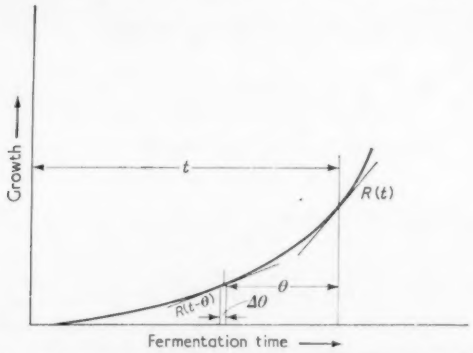

Fig. 2. Relation between growth rate and cell-age distribution

rate for the concentration of cells becoming of age θ at time t should be given by $R(t-\theta)$. Thus in a logarithmic growth phase if the growth rate is

$$R(t) = dC/dt = \alpha C_0 e^{\alpha t} \tag{10}$$

then the concentration of cells age θ per unit time at time t counting from the beginning of the phase is

$$C(\theta)_t = \alpha C_0 e^{\alpha(t-\theta)} \tag{11}$$

In a linear growth phase,

$$R(t) = dC/dt = R \tag{12}$$

consequently,

$$C(\theta)_t = R \tag{13}$$

Similarly, in a retarded growth phase, if the growth rate can be represented by

$$R(t) = dC/dt = \beta C_f e^{-\beta t} \tag{14}$$

then,

$$C(\theta)_t = \beta C_f e^{-\beta(t-\theta)} \tag{15}$$

The general equation describing the overall product accumulation covering all three phases shown in Fig. 3 is:

$$\begin{aligned} P = & \int_0^{T_2} \alpha C_0 e^{\alpha(T_2-\theta)} \int_0^{\theta} \sum_i A_i e^{-k_i\theta} d\theta d\theta \\ & - \int_0^{(T_2-t_2)} \alpha C_0 e^{\alpha(T_2-\theta)} \int_0^{\theta} \sum_i A_i e^{-k_i\theta} d\theta d\theta \\ & + \int_0^{T_3} R \int_0^{\theta} \sum_i A_i e^{-k_i\theta} d\theta d\theta - \int_0^{(T_3-t_3)} R \int_0^{\theta} \sum_i A_i e^{-k_i\theta} d\theta d\theta \\ & + \int_0^{t_4} \beta C_f e^{-\beta(t_4-\theta)} \int_0^{\theta} \sum_i A_i e^{-k_i\theta} d\theta d\theta \end{aligned} \tag{16}$$

where

$$\sum_i A_i \geq 0.$$

However, if there is a lag period of $\bar{a}$ for the product accumulation, the lower integration limits of equation (16) are replaced by $\bar{a}$. Under such circumstances, $\sum_i A_i < 0$.

In some process conditions, the propagation of an organism may not necessarily proceed through all these phases; often only one or two of the later phases are involved.

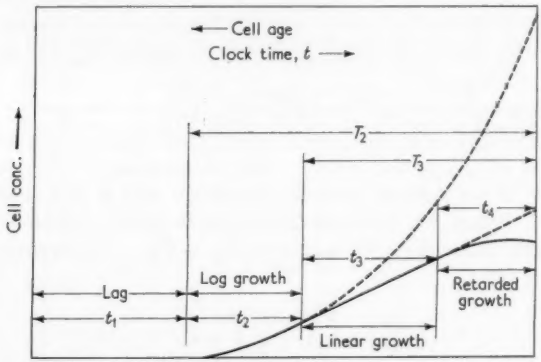

Fig. 3. Growth of micro-organism in batch process

Age Distribution in Continuous Processes

In continuous processes employing stirred cascade tanks, the age distribution in a cell population of any one tank may be obtained by making a material balance on cell concentration of the same age in the vessel under investigation. Thus,

$$\frac{\partial C_n(\theta)}{\partial \theta}+\frac{\partial C_n(\theta)}{\partial t} = R_n\delta(\theta)+\frac{F}{V_n}[C_{n-1}(\theta)-C_n(\theta)] \qquad (17)$$

where

$$\delta(\theta) = 0 \quad \text{when} \quad \theta \neq 0$$

and

$$\delta(\theta) = 1 \quad \text{when} \quad \theta = 0.$$

The function $\delta(\theta)$ is introduced to define the fact that all new growth starts out with age zero.

Under steady state conditions, i.e. $\partial C_n(\theta)/\partial t = 0$, equation (17) is reduced to

$$\frac{dC_n(\theta)}{d\theta} = R\delta(\theta)+\frac{F}{V_n}[C_{n-1}(\theta)-C_n(\theta)] \qquad (18)$$

Then the general solution of equation (18) is

$$C_n(\theta) = \mathcal{L}^{-1}\frac{R_n}{(\mathrm{p}+F/V_n)} + \frac{F}{V_n}\cdot\frac{R_{n-1}}{(\mathrm{p}+F/V_n)(\mathrm{p}+F/V_{n-1})} + \frac{F^2}{V_n\cdot V_{n-1}}\cdot\frac{R_{n-2}}{(\mathrm{p}+F/V_n)(\mathrm{p}+F/V_{n-1})(\mathrm{p}+F/V_{n-2})} + \cdots + \frac{F^{n-1}}{V_n\cdot V_{n-1}\cdots V_2}\cdot\frac{R_1}{(\mathrm{p}+F/V_n)(\mathrm{p}+F/V_{n-1})\cdots(\mathrm{p}+F/V_1)} \tag{19}$$

where $\mathcal{L}^{-1}$ is an inverse Laplace transform and p is a transform operator. When the volumes of culture in all the tanks are kept at the same value, i.e., $V_1 = V_2 = V_3 \cdots V_n = V$, equation (19) is reduced to:

$$C_n(\theta) = \left[R_n + R_{n-1}\left(\frac{F\theta}{V}\right) + \frac{R_{n-2}}{2!}\left(\frac{F\theta}{V}\right)^2 \cdots + \frac{R_1}{(n-1)!}\left(\frac{F\theta}{V}\right)^{n-1}\right]e^{-F\theta/V} \tag{20}$$

The general equation describing overall product concentration in the nth tank under steady state condition is

$$P_n = \int_0^\infty C_n(\theta)\int_0^\theta \sum_i A_i e^{-k_i\theta}d\theta d\theta \tag{21}$$

The lower integration limits are changed according to the lag time for models with a production lag period.

Examples and Discussion

Microbiological processes without the characteristics of production lag and the destruction of already formed product may be fitted with the model type represented by equation (7). Conversion of α-keto-adepic acid to lysine in baker's yeast as described by Jensen and Shu [10] serves as an excellent example of this type of model. Under their experimental conditions, the growth rate is kept almost constant from beginning to end. Hydrogen ion concentration and other environmental conditions are maintained in the range where the rate process is not significantly affected.

When the substrate, α-keto-adepic acid, is added to the culture at various stages of growth, the lysine production proceeds linearly for a period of time even after growth has been discontinued. The total amount of lysine formed is dependent on the time of addition even though an excess of α-keto-adepic acid remained at the end (see Fig. 4). A simple production model

$$r_p = 0{\cdot}0637e^{-0{\cdot}514\theta} \tag{22}$$

was found to satisfy the lysine process in all three examples within the range of experimental error. The cell age θ is counted in

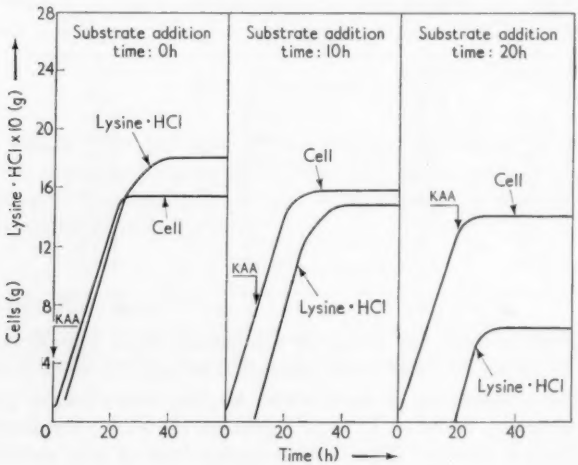

Fig. 4. Production of lysine by yeast

hours and r_p is expressed in terms of grams of lysine per gram of cell per hour. Data summarized in Table I illustrate the extent of agreement between experimental and predicted yields during the entire period of the process. In Table II, a comparison of predicted and experimental yields for experiments made with different substrate addition times is summarized.

In a study of lactic acid fermentation, Luedeking and Piret[11] established an empirical relation between overall lactic acid yield

and the growth of the culture:

$$\left.\frac{dP}{dt}\right|_t = \alpha' \left.\frac{dC}{dt}\right|_t + \beta' C_t \qquad (23)$$

for a batch process. This implies that there are two acid producing rate processes in operation. One is proportional to cell

Table I. Production of lysine-rich yeast. Test of fitness of prediction model in experiment with the substrate added at the beginning

Time, h	Experimental data: Yeast conc., g/l.	Experimental data: Lysine conc., g/l.	Predicted lysine conc., g/l.
0	0·9	0	0
5	2·2	0·15	0·22
10	5·9	0·52	0·55
15	10·6	0·83	0·89
20	13·0	1·25	1·23
25	15·3	1·74	1·57
30	16·2	1·64	1·75
35	15·0	1·70	1·77
40	15·7	1·76	1·77
45	15·0	1·82	1·77

concentration and the other is associated with the process of growth. Each of these rate processes, as may be seen from the following discussion, is equivalent to the overall rate process represented by a model of type equation (7) with very large and very small k values. The instantaneous rate of acid production at any time during the given growth phase in a batch process,

Table II. Production of lysine-rich yeast. Comparison of calculated and experimental yields for experiments with substrate added at various process periods

Substrate addition time, h	Experimental data, max. lysine conc.	Calculated value, max. lysine conc.
0	1·82	1·77
10	1·48	1·30
20	0·64	0·45

which proceeds according to equation (7), is

$$\left.\frac{dP}{dt}\right|_t = \int_0^t C(\theta)Ae^{-k\theta}d\theta = \int_0^t R(t-\theta)Ae^{-k\theta}d\theta \tag{24}$$

When k is very small, say $k \to 0$,

$$\left.\frac{dP}{dt}\right|_t = A\int_0^t C(\theta)d\theta = AC_t \tag{25}$$

Thus, the rate of production is proportional to the cell concentration in the vessel at the time of measurement. On the other hand, when k becomes very large, the value of $Ae^{-k\theta}$ is significant only when θ is very small, and $R(t-\theta)$ approaches $R(t)$ as θ assumes a very small value. Thus, equation (24) becomes

$$\left.\frac{dP}{dt}\right|_t = R(t)\int_0^t Ae^{-k\theta}d\theta = A'\left.\frac{dC}{dt}\right|_t \tag{26}$$

which means that the rate of the product formation is proportional to the rate of growth. Thus, the lactic acid production according to Luedeking and Piret is equivalent to the proposed general model representation:

$$r_p = A_1e^{-k_1\theta} + A_2e^{-k_2\theta} \tag{27}$$

with a relatively large value of k_1 and small value of k_2.

For processes in which the phase of product accumulation lags behind the cell multiplication and the product–cell relationships

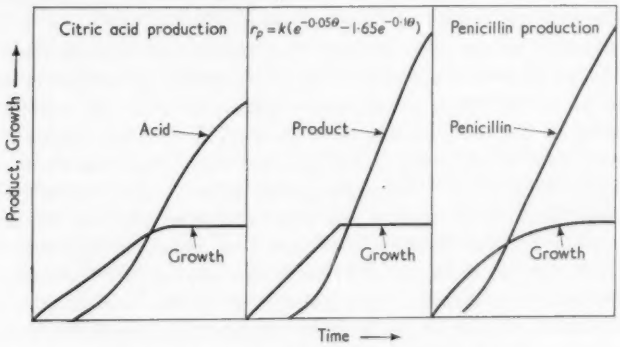

Fig. 5. Product accumulation with lag phase

are non-linear, the model type described by equations (8) and (9) would serve as best fit. Graphical illustration of such a model as compared with citric acid[12] and penicillin[13] processes is shown in Fig. 5. No attempt has been made to achieve a perfect fit in this demonstration. The phenomenon of transient product accumulation with production lag is illustrated in Fig. 6. A small

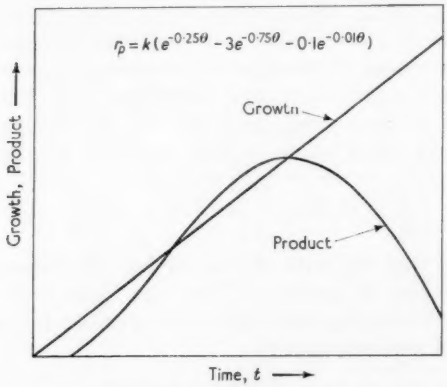

Fig. 6. Transient accumulation of a product

k value is used in a negative term of the model. These examples demonstrate the flexibility of the proposed model in describing various characteristics of product accumulation.

Prediction of the performance of a continuous culture method from batch process data can be made by applying the production model found in batch processes to equation (21). A non-linear relationship between growth and product formation presents no difficulty as it would occur if the problem were treated as a classical chemical reaction.[14] The discrepancy arises from the fact that in a microbiological process the reaction takes place in separate cells, while in the classical treatment of chemical reactors the reaction mixture is assumed to be a continuous single phase. A comparison between the proficiency of batch and continuous culture methods can be made on the basis of the constants used in the model. For instance, a process of model type represented by

equation (7) with a constant growth rate when carried out in a single-stage continuous culture tank is definitely less efficient than when it is carried out by a batch process of the same production capacity if the value kt is larger than 1·0; the reverse is true when the value is less than 0·6; and the value for an equivalent performance is somewhere between 1·0 and 0·6. This may be seen from direct comparison of the equations of product accumulation, i.e.

$$\Delta P = \int_0^\infty R_1 e^{-F\theta/V} \int_0^\theta A e^{-k\theta} d\theta d\theta - \int_0^t R_b \int_0^\theta A e^{-k\theta} d\theta d\theta \tag{28}$$

When $V/F = t$ and $R_1 = R_b$, equation (28) is reduced to

$$\begin{aligned} \Delta P &= -\left[\left(\frac{1}{t} - k\right)^{-1} - \frac{1}{k}(1 - e^{-kt})\right] \frac{RA}{k} \\ &= RAt^2\left(\frac{1}{2} - \frac{5}{6}kt + \frac{23}{24}(kt)^2 - \frac{119}{120}(kt)^3 - \cdots\right) \end{aligned} \tag{29}$$

For values of $kt < 0{\cdot}6$, $\Delta P > 0$; and for $kt > 1$, $\Delta P < 0$.

Again, the idea of a production model offers a convenient interpretation for the relatively poor performance of some continuous fermentations which the inherent short-circuit phenomenon alone cannot account for.

In the above discussion, consideration is given only to the case of a constant environmental condition. It is well known that in the process of growing a culture usually more than one product is formed. These products may interfere with the production of one other. Thus, in batch processes, the production model is often modified by these environmental changes and the production pattern may change with time. Such a feed-back interfering phenomenon is best demonstrated by the pH change and its effect on the formation of a principle product under investigation. The pH change may be considered as a production of hydrogen or hydroxyl ions and its accumulation pattern may be described by using a suitable model in the general equation (16). If the quantitative effect of pH on the model constants is known, it is possible to take the pH perturbation into consideration. This rigorous line of treatment should be applicable to other environmental changes primarily caused by the organism itself.

Although the mechanism of the product accumulation may be obscure, the present postulate is attractive because of its unified concept which is applicable to a wide variety of microbiological problems including batch processes as well as continuous processes and environmental drifts. The use of an exponential decay function as the basic building block for the model synthesis is consistent with general ideas of the aging processes occurring in nature. The assumption of a delay in product formation in an individual cell is also compatible with the idea of functional variations with physiological age in biological materials, and the general belief that a time lag is required to accumulate necessary intermediates and enzymes for certain product syntheses. With the present-day knowledge of simulation and computer techniques, the labour involved in computation should no longer impose obstacles to this type of approach to biological problems.

Acknowledgments. The author wishes to acknowledge the helpful discussions offered by Dr. T. M. Kuo and Mr. R. W. Letts of the Production Analysis Section of American Cyanamid Company.

Notation

$A_1, A_2, A_3, \ldots A_i$ = coefficients of decay functions of the production model.

C, C_0, C_t, C_f = concentration of cells in general, overall initial concentration of cells, concentration of cells at time t and concentration of cells at the end of the process, respectively.

$C(\theta)$ = concentration of cells age θ per unit time.

$C_n(\theta)$ = concentration of cells age θ per unit time in nth tank of a continuous process.

$C(\theta)_t$ = concentration of cells age θ per unit time at time t in a batch process.

F = overflow volume rate in continuous fermentor.

$k, k_1, k_2, \ldots k_i$ = decay constants for the respective terms in the production model.

p_θ = quantity of product formed per unit weight of a single cell of age θ.

P, P_t, P_n = overall production, overall product accumulated at time t, and product accumulated in nth fermentor, respectively.

r_p = rate of product accumulation by unit weight of cell of age θ.

$R, R_b, R(t), R_n$ = linear growth rate, linear growth rate in a batch process, growth rate at time t and growth rate in nth tank, respectively.

$t, t_1, t_2, \ldots T$ = fermentation times according to Fig. 3.
$V_1, V_2, \ldots V_n$ = culture volume in 1st, 2nd, . . . nth tank, respectively.
α, β = constants for exponential growth equations.
α', β' = proportional constants in lactic acid production equation after Luedeking and Piret.
A' = a constant.
θ = cell age.
i = ith term in production model.
n = nth tank in continuous process.

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Note

An Anti-foam Low-Temperature Laboratory Evaporator

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The need for an efficient and simple laboratory, low-temperature evaporator has prompted the design of several types of concentrator. Many of these are efficient, but do not combine the features included in the present design. The two models described have no moving parts, are compact, simple to build and operate, will evaporate foaming material with little difficulty, and can be dismantled easily for cleaning.

The large model shown in Fig. 1 consists of an evaporating

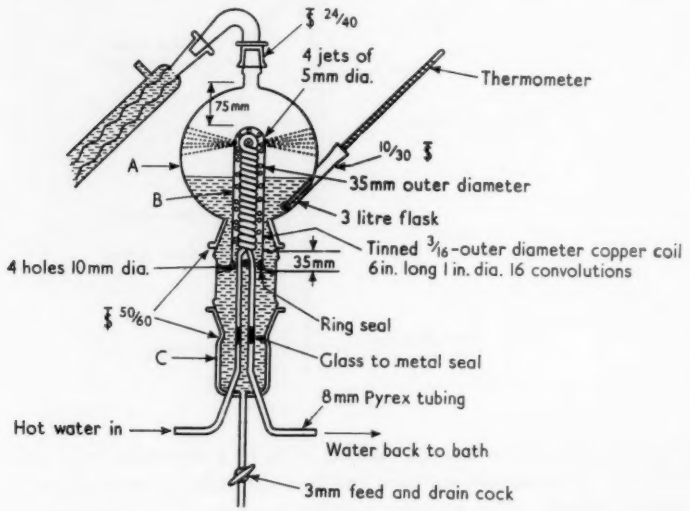

Fig. 1

chamber (A), a heating and jet section (B), a heating coil section (C), and a condenser connected to a vacuum pump. The liquid to be evaporated is heated in section B by the heating coil. The boiling liquid is forced (by the increase in vapour pressure) through the four jets at the top of the heating section onto the walls of the evaporating chamber (A). This feature breaks any foam present and forms a liquid film on the surface of the evaporating chamber. The liquid in section A is returned to the heating section through the four holes around the ring-seal at the base of the heating and jet section (B).

The smaller model (Fig. 2), made with material readily available in the laboratory, is advantageous when an all-glass apparatus is

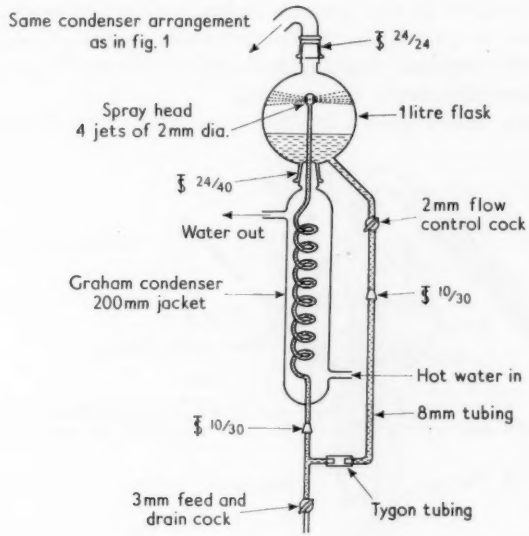

Fig. 2

required and also when the sample is to be evaporated to a small volume. The sample solution can be evaporated down to approximately 20 ml. The heating section consists of a Graham condenser which has been modified to incorporate the spray-jets.

Re-circulation of the liquid from the evaporating chamber to the heating section takes place through a side tube. In other respects, the model resembles the larger type already described.

For a trouble-free 'start-up', one third of chamber A is slowly filled with the sample solution through the feed cock by means of vacuum. Once most of the air has bubbled out of the liquid, and with the apparatus under vacuum, the hot water is circulated in the heating section.

The capacity of evaporation for the large model is approximately 1 l./h at 30°C with a pressure of 30 mm of Hg, and the heating water at 50°C. The small model will evaporate 250 ml water/h at 22°C, with a pressure of 20 mm of Hg, and the water in the heating jacket at 45°C. These evaporators have been used successfully with milk, milk serum, and alcohol extracts of bacterial cells.

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Note

Percentage Estimation of Denitrifying Bacteria

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While investigating the occurrence of denitrifying bacteria in sewage effluents by means of the most probable number method, difficulty was experienced in assessing the percentage of these in relation to the total bacterial population. An attempt was therefore made to express the total number of bacteria in most probable numbers instead of total plate count which gave results that seemed to be too low. The most probable number of bacteria is denoted by M.P.N. Except for modifications as described, procedures were as given in 'Standard Methods'.[1]

Incubation of nitrate broth at 37 °C for 14 days gave the highest reading of positive tubes. This period was, however, too long. Seven days' incubation was found to be practical and very few tubes turned positive after this period. All readings for M.P.N. were consequently taken after an incubation period of seven days.

The total bacterial population was estimated in two different ways, viz. total plate count on nutrient agar with incubation at 37 °C for 48 h and M.P.N. after inoculation into nutrient broth and incubation for seven days at 37 °C as in the case of nitrate and nitrite broth. Longer periods of incubation of plates led to drying out. All tubes showing any sign of growth were taken as positive. Nitrate and nitrite reducing bacteria were estimated by inoculating maturation pond samples into nutrient broth containing 0·1 per cent potassium nitrate and 0·5 per cent potassium nitrite respectively. Gas production in either of these media was used as the criterion in the estimation of reducing ability. All the conditions for the various M.P.N. estimations were exactly similar and therefore comparable.

Water samples were taken from the influent and effluent of two

Table I. Most probable number of nitrate and nitrite reducing bacteria and comparison of total population of bacteria estimated by means of M.P.N. and total plate count in maturation pond influent and effluent water

Sampling date	Most probable number of bacteria per ml			Total plate count, no. of bacteria/ml	Denitrifying bacteria expressed as % of total plate count and total M.P.N.			
					% of total plate count		% of total M.P.N.	
	Nitrate reducing bacteria	Nitrite reducing bacteria	Total number of bacteria	Total number of bacteria	NO_3 reducers	NO_2 reducers	NO_3 reducers	NO_2 reducers
				Pond Influent				
13/5/59	3,300,000	2,300,000	64,000,000	3,930,000	84·0	58·5	5·2	3·6
26/5/59	93,000	68,000	4,900,000	860,000	10·8	7·9	1·9	1·4
2/6/59	920,000	540,000	14,000,000	720,000	127·8	75·0	6·6	3·9
16/6/59	46,000	22,000	1,300,000	235,000	19·6	9·4	3·5	1·7
24/6/59	140,000	26,000	3,400,000	240,000	58·3	10·8	4·1	0·8
30/6/59	46,000	70,000	4,900,000	92,000	50·0	76·1	0·9	1·4
14/7/59	350,000	280,000	92,000,000	892,000	39·2	31·4	0·4	0·3
21/7/59	79,000	170,000	22,000,000	480,000	16·5	35·4	0·4	0·8
28/7/59	920,000	27,000	2,200,000	390,000	235·9	6·9	41·8	1·2
11/8/59	280,000	220,000	9,200,000	1,086,000	25·8	20·3	3·0	2·4
Geometrical mean	238,500	125,300	9,101,000	544,500	43·8	23·0	2·6	1·4
				Pond I Effluent				
13/5/59	2,000	2,000	140,000	63,000	3·2	3·2	1·4	1·4
26/5/59	11,000	1,700	920,000	93,000	11·8	1·8	1·2	0·2
2/6/59	35,000	24,000	350,000	40,500	86·4	59·3	10·0	6·9
16/6/59	54,000	54,000	490,000	81,000	66·7	66·7	11·0	11·0
24/6/59	92,000	3,400	2,200,000	371,000	24·8	0·9	4·2	0·2
30/6/59	18,000	3,400	5,400,000	298,000	6·0	1·1	0·3	0·1
14/7/59	92,000	24,000	2,200,000	460,000	20·0	5·2	4·2	1·1
21/7/59	22,000	35,000	5,400,000	1,230,000	1·8	2·8	0·4	0·6
28/7/59	35,000	54,000	3,500,000	210,000	16·7	25·7	1·0	1·5
11/8/59	17,000	2,800	1,600,000	561,300	3·0	0·5	1·1	0·2
Geometrical mean	24,650	9,574	1,331,000	206,600	11·9	4·6	1·8	0·6

experimental maturation ponds in Pretoria. The influent for both ponds was the same and consisted of partly purified sewage effluent from a humus tank which had previously passed through a biological filter. Samples were collected in 250-ml glass-stoppered sample bottles and the same series of dilution bottles was used to inoculate both plates and tubes.

Some of the results obtained are given in Table I. There is a large difference between the total numbers of bacteria obtained by means of the two different methods. The percentage of nitrate and nitrite reducers as expressed in terms of the total plate count is obviously too high. A more realistic value is obtained by using the M.P.N. method for estimating the total bacterial population.